

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/82, 9/10</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/34490</b> <b>(43) International Publication Date:</b> 15 June 2000 (15.06.00)
<b>(21) International Application Number:</b> PCT/JP99/06881 <b>(22) International Filing Date:</b> 8 December 1999 (08.12.99)  <b>(30) Priority Data:</b> 10/350584 9 December 1998 (09.12.98) JP  <b>(71)(72) Applicants and Inventors:</b> SEKI, Tatsuji [JP/JP]; 5-13-53-209, Mino, Mino-shi, Osaka 562-0001 (JP). FU- JIYAMA, Kazuhito [JP/JP]; A18-308, 1-28, Yamadanishi, Suita-shi, Osaka 565-0824 (JP).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> YOSHIDA, Toshiomi [JP/JP]; A1-505, 2-4, Yamadanishi, Suita-shi, Osaka 565-0824 (JP).  <b>(74) Agent:</b> YAMAMOTO, Shusaku; Crystal Tower, 15th Floor, 2-27, Shiromi 1-chome, Chuo-ku, Osaka-shi, Osaka 540-6015 (JP).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> A METHOD FOR MANUFACTURING GLYCOPROTEINS HAVING HUMAN-TYPE GLYCOSYLATION  <b>(57) Abstract</b>  The present invention provides a method for manufacturing a glycoprotein having a human-type sugar chain comprising a step in which a transformed plant cell is obtained by introducing to a plant cell the gene of glycosyltransferase and the gene of an exogenous glycoprotein, and a step in which the obtained transformed plant cell is cultivated.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## DESCRIPTION

## A METHOD FOR MANUFACTURING GLYCOPROTEINS HAVING HUMAN-TYPE GLYCOSYLATION

TECHNICAL FIELD

- 5 The present invention relates to expression of exogenous glycoproteins by plants.

BACKGROUND ART

- 10 Many of the functional proteins in living organisms are glycoproteins. It has been elucidated that the diversity of the sugar chains in glycoproteins play several important roles physiologically (Lain, R.A., Glycobiology, 4, 759-767, 1994).
- 15 In recent years, it has also become clear that the action of sugar chains can be divided into two categories. In the first case, sugar chains have a direct function as ligands for binding cells, or as receptors for bacteria and viruses, in the clearance of glycoproteins from the blood, lysosome
- 20 targeting of lysosome enzymes and the targeting by glycoproteins toward specific tissues and organs. For example, the contribution of glycoprotein sugar chains in the infection of target cells by the AIDS virus (HIV) has been established (Rahebi, L. et al., Glycoconj. J., 12, 7-16,
- 25 1995). The surface of HIV is covered with envelope protein gp120. The binding of gp120 sugar chains to the CD4 of target cells is the beginning of infection by the HIV virus. In the second case, the sugar chain itself is not the functional molecule but indirectly contributes to the formation of the
- 30 higher-order structure of proteins, solubility of proteins, protease resistance of proteins, inhibition of antigenicity, protein function modification, protein regeneration rate adjustment, and adjustment of the amount of proteins

expressed in cell layers. For example, sugar chains are instrumental in the adjustment of the adhesion of nerve cell adhesion molecules which are distributed widely in the nervous system (Edelman, G.M., Ann. Rev. Biochem., 54, 135-169, 1985).

In eukaryotes, glycoprotein sugar chains are synthesized on lipids of the Endoplasmic reticulum as precursor sugar chains. The sugar chain portion is transferred to the protein, then some of the sugar residues on the protein are removed in the Endoplasmic reticulum, and then the glycoprotein is transported to Golgi bodies. In the Golgi bodies, after the excess sugar residues have been removed, further sugar residues (e.g. mannose) are added and the sugar chain is extended (Narimatsu, H., Microbiol. Immunol., 38, 489-504, 1994).

More specifically, for example, Glc3Man9GlcNAc2 on dolichol anchors is transferred to protein in the ER membrane (Moremen K.W., Trimble, R.B. and Herscovics A., *Glycobiology* 1994 Apr;4(2):113-25, Glycosidases of the asparagine-linked oligosaccharide processing pathway; and Sturm, A. 1995 N-Glycosylation of plant proteins. In: New Comprehensive Biochemistry. Glycoproteins, Vol.29a., Montreuil, J., Schachter, H. and Vliegenthart, J.F.G.(eds). Elsevier Science Publishers B.V., The Netherlands, pp. 521-541). ER-glucosidase I and II removes three glucose units (Sturm, A. 1995, *supra*; and Kaushal G.P. and Elbein A.D., 1989, Glycoprotein processing enzymes in plants. In *Methods Enzymology* 179, Complex Carbohydrates Part F. Ginsburg V. (ed), Academic Press, Inc. NY, pp.452-475). The resulting high mannose structure (Man9GlcNAc2) is trimmed by ER-mannosidase (Moremen K.W. et al, *supra*,; and Kornfeld, R.

and Kornfeld, S., *Annu. Rev. Biochem.* 54, 631-664, 1985; Assembly of asparagine-linked oligosaccharides). The number of mannose residues removed varies according to the differences in the accessibility to the processing enzymes.

5 The isomers Man8-, Man7-, Man6- and Man5GlcNAc2 are produced during processing by ER-mannosidase and Mannosidase I (Kornfeld, R. and Kornfeld, S., *supra*). When four mannose residues are completely removed by Mannosidase I (Man I), the product is Man5GlcNAc2. N-acetylglucosaminyl

10 transferase I (GlcNAc I) transfers N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to Man5GlcNAc2, resulting in GlcNAcMan5GlcNAc2 (Schachter, H., Narasimhan, S., Gleeson, P., and Vella, G., *Glycosyltransferases involved in elongation of N-glycosidically linked oligosaccharides of the complex or N-acetylgalactosamine type. In: Methods*

15 *Enzymol* 98: Biomembranes Part L. Fleischer, S., and Fleischer, B. (ed), Academic Press, Inc. NY, pp.98-134 pp. 98-134, 1983). Mannosidase II (Man II) removes two mannose residues from GlcNAcMan5GlcNAc2, yielding

20 GlcNAcMan3GlcNAc2 (Kaushal, G.P. and Elbein, A.D., *supra*; and Kornfeld, R. and Kornfeld, S., *supra*). The oligosaccharide GlcNAcMan4GlcNAc2 is used as a substrate of N-acetylglucosaminyl transferase II (GlcNAc II) (Moremen K.W. et al, *supra*; Kaushal, G.P. and Elbein, A.D., *supra*; and Kornfeld, R. and Kornfeld, S., *supra*). FIG 19 summarizes

25 the above described structures of N-linked glycans and enzymes involved in sugar chain modification pathway in the Endoplasmic reticulum and Goldi bodies. In FIG 19,  $\diamond$  denotes glucose,  $\square$  denotes GlcNAc,  $\circ$  denotes mannose,  $\bullet$  denotes galactose, and  $\blacksquare$  denotes sialic acid, respectively.

30

The sugar addition in the Golgi bodies is called terminal sugar chain synthesis. The process differs widely among

living organisms. The sugar chain synthesis depends on the type of eukaryote. The resulting sugar chain structure is species-specific, and reflects the evolution of sugar adding transferase and the Golgi bodies (Narimatsu, H., Cellular Biology, 15, 802-810, 1996).

Regarding asparagine-linked (N-linked) sugar chains; in animals, there are high mannose-type sugar chains, complex-type sugar chains and hybrid-type sugar chains. These structures are shown in FIG 1. The complex-type sugar chains in plants have  $\alpha$ 1,3 fucose and  $\beta$ 1,2 xylose which are sugar residues that are not found in animals (Johnson, K.D. and Chrispeels, M.J., Plant Physiol., 84, 1301-1308, 1997, Kimura, Y. et al., Biosci. Biotech. Biochem., 56, 215-222, 1992). In the case of N-linked sugar chains, sialic acid has been found in animal sugar chains but has not been found in plant sugar chains. Regarding galactose, which is generally found in animal sugar chains, although the presence thereof has been found in some plant sugar chains (Takahashi, N. and Hotta, T., Biochemistry, 25, 388-395, 1986), the examples thereof are few. The linkage-type thereof is a  $\beta$ 1,3 linkage (FEBS Lett 1997 Sep 29, 415(2), 186-191, Identification of the human Lewis(a) carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium mytillus* L.), Melo NS, Nimtz M, Contradt HS, Fevereiro PS, Costa J; Plant J. 1997 Dec. 12(6), 1411-1417, N-glycans harboring the Lewis a epitope are expressed at the surface of plant cells., Fitchette-Laine AC, Gomord V, Cabanes M, Michalski JC, Saint Macary M, Foucher B, Cavelier B, Hawes C, Lerouge P, Faye L). This linkage is different from those found in animals.

Glycoproteins derived from humans include human

erythropoietin (EPO). In order to produce glycoproteins with sugar chain structures similar to humans, these glycoproteins are produced in animal host cells. However, EPO produced in animal cells has a sugar chain structure that is different from the natural human sugar chain structure. As a result, in vivo activity of EPO is reduced (Takeuchi, M. et al., Proc. Natl. Acad. Sci. USA, 86, 7819-7822, 1989). The sugar chain structure in other proteins derived from humans, such as hormones and interferon, have also been analyzed and manufactured with the same glycosylation limitations.

The methods used to introduce exogenous genes to plants include the *Agrobacterium* method (Weising, K. et al., Annu. Rev. Genet., 22, 421, 1988), the electroporation method (Toriyama, K. et al., Bio/Technology, 6, 1072, 1988), and the gold particle method (Gasser, C.G. and Fraley, R.T., Science, 244, 1293, 1989). Albumin (Sijmons, P.C. et al., Bio/Technology, 8, 217, 1990), enkephalin (Vandekerckhove, J. et al., Bio/Technology, 7, 929, 1989), and monoclonal antibodies (Benvenuto, E. et al., Plant Mol. Biol., 17, 865, 1991 and Hiatt, A. et al., Nature, 342, 76, 1989) have been manufactured in plants. Hepatitis B virus surface antigens (HBsAg) (Mason, H.S. et al., Proc. Natl. Acad. Sci. USA, 89, 11745, 1992) and secretion-type IgA (Hiatt, A. and Ma, J.S.K., FEBS Lett., 307, 71, 1992) have also been manufactured in plant cells. However, when human-derived glycoproteins are expressed in plants, the sugar chains in the manufactured glycoproteins have different structures than the sugar chains in the glycoproteins produced in humans because the sugar adding mechanism in plants is different from the sugar adding mechanism in animals. As a result, glycoproteins do not have the original physiological

activity and may be immunogenic in humans (Wilson, I.B.H. et al., Glycobiol., Vol. 8, No. 7, pp. 651-661, 1998).

#### DISCLOSURE OF THE INVENTION

5 The purpose of the present invention is to solve the problems associated with the prior art by providing plant-produced recombinant glycoproteins with mammalian, e.g., human-type sugar chains.

10 The present invention is a method of manufacturing a glycoprotein having a human-type sugar chain comprising a step in which a transformed plant cell is obtained by introducing to a plant cell the gene of an enzyme capable of conducting a transfer reaction of a galactose residue  
15 to a non-reducing terminal acetylglucosamine residue and the gene of a exogenous glycoprotein, and a step in which the obtained transformed plant cell is cultivated.

In the present invention, the glycoprotein with a human-type  
20 sugar chain can comprise a core sugar chain and an outer sugar chain, the core sugar chain consists essentially of a plurality of mannose and acetylglucosamine, and the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.

25 In the present invention, the outer sugar chain can have a straight chain configuration or a branched configuration. In the present invention, the branched sugar chain portion can have a mono-, bi-, tri- or tetra configuration.  
30 In the present invention, the glycoprotein can contain neither fucose nor xylose.

The present invention is also a plant cell having a sugar



chain adding mechanism which can conduct a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue, wherein the sugar chain adding mechanism acts on a sugar chain containing a core sugar chain and an outer sugar chain, wherein the core sugar chain consists essentially of a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.

10 In the present invention, a glycoprotein with a human-type sugar chain is obtained using this method.

#### BRIEF DESCRIPTION OF DRAWINGS

15 FIG 1. A schematic drawing of typical N-linked sugar chain configurations.

FIG 2. Schematic drawings of the cloning method for hGT.

FIG 3. Schematic drawings of the method used to construct vector pGAhGT for hGT expression.

20 FIG 4. A photograph showing a Southern blot analysis of a genome of cultivated transformed tobacco cells. FIG 4 (A) shows electrophoresis after the genome DNA (40  $\mu$ g) has been digested by EcoRI and HindIII. The numbers at the left indicate the position of the DNA molecular weight marker.

25 FIG 4 (B) shows a schematic drawing of a 2.2 kb fragment containing a promoter, hGT and terminator, which is integrated into the transformed cell.

30 FIG 5. FIG 5 is a photograph of the Western blotting of immunoreactive protein from transformed tobacco BY2 cells (WT) and wild type tobacco BY2 cells (WT). The protein was denatured, electrophoresed on 10% SDS-PAGE, and then transferred electrically to nitrocellulose film. The samples were as follows: lane 1 = GT1 cell extract; lane

2 = GT 6 cell extract; lane 3 = GT8 cell extract; lane 4  
= GT9 cell extract; lane 5 = wild type cell extract; lane  
6 = GT1 microsome fragment; lane 7 = GT6 microsome fragment;  
lane 8 = GT8 microsome fragment; lane 9 = GT9 microsome  
fragment; lane 10 = wild type microsome fragment.

5  
FIG 6. An electrophoresis photograph showing the detection  
of galactosylated glycoprotein using *Ricinus communis*  
(RCA<sub>120</sub>) affinity chromatography. The electrophoresed gel  
was visualized by silver staining. Lanes 1 and 2 show the  
10 protein from wild type BY2 cells, while Lanes 3 and 4 show  
the protein from transformed GT6 cells. The molecular  
weight is in KDa units.

15  
FIG 7. A photograph of Western blotting a showing the  
detection of galactosylated glycoprotein using *Ricinus*  
*communis* (RCA<sub>120</sub>) affinity chromatography. After the  
electrophoresed gel had been blotted on a nitrocellulose  
membrane, this membrane was visualized by lectin (RCA<sub>120</sub>)  
staining. Lanes 1 and 2 show the protein from a wild type  
BY2 cell, while Lanes 3 and 4 show the protein from  
20 transformed GT6 cells. The molecular weight is in KDa.

FIG 8. A photograph of a blotting in which the  
galactosylated glycoprotein from *Ricinus communis* (RCA<sub>120</sub>)  
affinity chromatography was probed with an antiserum  
specific to xylose in complex-type plant glycans. Lanes 1  
25 and 2 show the total protein extracts from BY2 and GT6,  
respectively, and Lane 3 shows the glycoprotein from GT6  
after RCA<sub>120</sub> affinity chromatography. The molecular weight  
is in KDa units.

30  
FIG 9. A schematic drawing of a plasmid pBIHm-HRP which is  
a binary vector with a kanamycin-resistant gene and a  
hygromycin-resistant gene, and has a HRP cDNA.

FIG 10. Photographs of isoelectric focusing and Western  
blotting which show HRP production in a suspension culture

of transgenic cells. FIG 10 (A) shows the results of isoelectric focusing and FIG 10 (B) shows the results of Western blotting. The abbreviations are as follows: WT = wild-type; BY2 - HRP 1, 5 and 7 = the clone numbers for BY2 cells transformed with a HRP gene; and GT-6 - HRP 4, 5 and 8 = the clone numbers for GT6 cells transformed with a HRP gene.

FIG 11. A graph showing the reverse-phase HPLC pattern of a PA sugar chain eluted in 0-15% acetonitrile linear gradient in 0.02% TFA over 60 minutes and at a flow rate of 1.2 ml/min. I-XI shows the fractions eluted and purified from size-fractionation HPLC. Excitation wavelength and emission wavelength were 310nm and 380nm, respectively.

FIG 12. Graphs showing the size-fractionation HPLC pattern of the PA sugar chain in FIG 11. Elution was performed in a 30-50% water gradient in the water-acetonitrile mixture over 40 minutes and at a flow rate of 0.8 ml/min. The excitation wavelength and emission wavelength were 310 nm and 380 nm, respectively.

FIG 13. A graph showing the elution position of peak-K2 on reverse phase HPLC wherein two standard sugar chain products A and B are compared with the peak K2. The elution conditions were the same as in FIG 11. That is, elution was performed in 0-15% acetonitrile linear gradient in 0.02% TFA over 60 minutes and at a flow rate of 1.2 ml/min.

FIG 14. Graphs showing the SF-HPLC profiles of galactosylated PA sugar chains obtained after exoglycosidase digestion. Elution was performed in a 30-50% water gradient in the water-acetonitrile mixture over 25 minutes and at a flow rate of 0.8 ml/min. (A) PA-sugar chain K-2: I is the elution position of the galactosylated PA sugar chain used; II is  $\beta$ -galactosidase digests of I; III is a N-acetyl- $\beta$ -D-glucosaminidase digests of II; IV is

jack bean  $\alpha$ -mannosidase digests of III. (B) PA-sugar chain  
L: I is the elution position of the galactosylated PA sugar  
chain used; II is  $\beta$ -galactosidase digests of I; III is  
N-acetyl- $\beta$ -D-glucosaminidase digests of II; IV is  $\alpha$ 1,2  
mannosidase digests of III; V is jack bean  $\alpha$ -mannosidase  
5 digests of III.

FIG 15. Estimated structures of the N-linked glycans  
obtained from the transformed cells. The numbers in the  
parentheses indicate the molar ratio.

10 FIG 16. Photographs of *Ricinus communis* 120 agglutinin  
(RCA<sub>120</sub>) affinity chromatography showing the detection of  
glycosylated HRP. FIG 16 (A) shows the results from silver  
staining, and FIG 16 (B) shows the results from lectin RCA<sub>120</sub>  
15 staining. The lectin-stained filter was cut into strips and  
then probed using lectin RCA<sub>120</sub> pre-incubated with buffer  
alone (I and II) or incubated in buffer with excess galactose  
(III). In (II), HRP was treated with  $\beta$ -galactosidase from  
*Diplococcus pneumoniae* before SDS-PAGE. Lane 1 is a  
20 collected fraction containing BY2-HRP and Lane 2 is a  
collected fraction containing GT6-HRP. The numbers to the  
left refer to the location and the size (KDa) of the standard  
protein.

FIG 17. A graph showing the results of reverse-phase HPLC  
of the PA sugar chains from purified HRP after RCA<sub>120</sub> affinity  
25 chromatography.

FIG 18. Photographs of Western blotting showing immune  
detection of plant specific complex-type glycans. The  
purified HRP is fractioned by SDS-PAGE, transferred to  
nitrocellulose, and confirmed with rabbit anti-HRP (A) and  
30 an antiserum which is specific for complex-type glycans of  
plants (B). Lane 1 = galactosylated HRP from GT6-HRP after  
RCA<sub>120</sub> affinity chromatography; Lane 2 = purified HRP from  
BY2-HRP. The position of the molecule size marker is shown

to the left in kDa. The galactosylated N-glycan on HRP derived from the transformant GT6-HRP cells did not react with an antiserum which has been shown to specifically react with  $\beta$ 1,2 xylose residue indicative of plant N-glycans.

5 FIG 19. Structures of N-linked glycans and enzymes involved in the sugar chain modification pathway in Endoplasmic reticulum and Golgi bodies.  $\diamond$  denotes glucose,  $\square$  denotes GlcNAc,  $\bigcirc$  denotes mannose,  $\bullet$  denotes galactose, and  $\blacksquare$  denotes sialic acid, respectively.

10 FIG 20. Structures of N-linked glycans and the ratio of each N-linked glycan in GT6 cell line along with those in wild-type BY2 cell line determined similarly.  $\square$  denotes GlcNAc,  $\bigcirc$  denotes mannose,  $\bullet$  denotes galactose, and  $\blacksquare$  denotes sialic acid, respectively.

15 FIG 21 illustrates one of the embodiment of the present invention. In GT6 cell line, the isomers Man7-, Man6- and Man5GlcNAc2 were observed. Because those high-mannose type oligosaccharides will be converted by some glycan processing enzymes to be substrates for  $\beta$ 1,4-galactosyltransferase (Gal T), introduction of GlcNAc I, Man I and Man II cDNAs  
20 could more efficiently lead the oligosaccharide Man7-5GlcNAc2 to GlcNAcMan3GlcNAc2, which can be a substrate of GalT.

FIG 22 also illustrates another the embodiment of the present invention. 1,4-Galactosyltransferase (Gal T) uses UDP-galactose as a donor substrate and GlcNAc2Man3GlcNAc2 as an acceptor substrate. Efficient supply of UDP-galactose will enhance the Gal T enzyme reaction and more galactosylated oligosaccharide will be produced.

30

#### BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be described in further detail. In performing the present invention,

unless otherwise indicated, any conventional technique can be used. These include methods for isolating and analyzing proteins as well as immunological methods. These methods can be conducted by using commercial kits, antibodies and markers.

The method of the present invention relates to a method of manufacturing glycoproteins with human-type sugar chains. In this specification, "human-type sugar chain" refers to a sugar chain with a galactose residue linked to a N-acetylglucosamine residue. The galactose residue in the human-type sugar chain can be the terminal sugar chain or a sialic acid residue can be linked to the outside of the galactose residue. Preferably, the glycoprotein of the present invention at least has no xylose or fucose linked to one or more of the following portions: the core sugar chain portion, the branched sugar chain portion, or the terminal sugar chain portion of the human-type sugar chain. More preferably, neither xylose or fucose should be linked to any portion of the human-type sugar chain, and ideally there should be no xylose or fucose contained in the human-type sugar chain at all.

The plant cells can be any plant cells desired. The plant cells can be cultured cells, cells in cultured tissue or cultured organs, or cells in a plant. Preferably, the plant cells should be cultured cells, or cells in cultured tissue or cultured organs. Most preferably, the plant cells should be cells in whole plants, or portions thereof, that produce glycoproteins with human-type sugar chains. The type of plant used in the manufacturing method of the present invention can be any type of plant that is used in gene transference. Examples of types of plants that can be used

in the manufacturing method of the present invention include plants in the families of *Solanaceae*, *Poaceae*, *Brassicaceae*, *Rosaceae*, *Leguminosae*, *Curcubitaceae*, *Lamiaceae*, *Liliaceae*, *Chenopodiaceae* and *Umbelliferae*.

5

Examples of plants in the *Solanaceae* family include plants in the *Nicotiana*, *Solanum*, *Datura*, *Lycopersicon* and *Petunia* genera. Specific examples include tobacco, eggplant, potato, tomato, chili pepper, and petunia.

10

Examples of plants in the *Poaceae* family include plants in the *Oryza*, *Hordenum*, *Secale*, *Saccharum*, *Echinochloa* and *Zea* genera. Specific examples include rice, barley, rye, *Echinochloa crus-galli*, sorghum, and maize.

15

Examples of plants in the *Brassicaceae* family include plants in the *Raphanus*, *Brassica*, *Arabidopsis*, *Wasabia*, and *Capsella* genera. Specific examples include Japanese white radish, rapeseed, *Arabidopsis thaliana*, Japanese horseradish, and *Capsella bursa-pastoris*.

20

Examples of plants in the *Rosaceae* family include plants in the *Orunus*, *Malus*, *Pynus*, *Fragaria*, and *Rosa* genera. Specific examples include plum, peach, apple, pear, Dutch strawberry, and rose.

25

Examples of plants in the *Leguminosae* family include plants in the *Glycine*, *Vigna*, *Phaseolus*, *Pisum*, *Vicia*, *Arachis*, *Trifolium*, *Alfalfa*, and *Medicago* genera. Specific examples include soybean, adzuki bean, kidney beans, peas, fava beans, peanuts, clover, and alfalfa.

30

Examples of plants in the *Curcubitaceae* family include

plants in the *Luffa*, *Curcubita*, and *Cucumis* genera. Specific examples include gourd, pumpkin, cucumber, and melon.

5      Examples of plants in the *Lamiaceae* family include plants in the *Lavandula*, *Mentha*, and *Perilla* genera. Specific examples include lavender, peppermint, and beefsteak plant.

10      Examples of plants in the *Liliaceae* family include plants in the *Allium*, *Lilium*, and *Tulipa* genera. Specific examples include onion, garlic, lily, and tulip.

15      Examples of plants in the *Chenopodiaceae* family include plants in the *Spinacia* genera. A specific example is spinach.

20      Examples of plants in the *Umbelliferae* family include plants in the *Angelica*, *Daucus*, *Cryptotaenia*, and *Apitum* genera. Specific examples include Japanese udo, carrot, honewort, and celery.

25      Preferably, the plants used in the manufacturing method of the present invention should be tobacco, tomato, potato, rice, maize, radish, soybean, peas, alfalfa or spinach. Ideally, the plants used in the manufacturing method of the present invention should be tobacco, tomato, potato, maize or soybean.

30      In this specification, "an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue" refers to an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine



residue produced when a sugar chain is added after synthesis of the protein portion of the glycoprotein in the plant cell. Specific examples of these enzymes include galactosyltransferase, galactosidase, and  $\beta$ -galactosidase. 5 These enzymes can be derived from any animal desired. Preferably, these enzymes should be derived from a mammal, and ideally these enzymes should be derived from a human.

10 In this specification, "the gene of an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue" can be a gene which can be isolated from an animal cell using a nucleotide sequence of an encoded enzyme well known in the art, or commercially available genes altered for expression 15 in plants.

In this specification, "gene" usually refers to the structural gene portion. A control sequence such as a promoter, operator and terminator can be linked to the gene 20 so as to properly express the gene in a plant.

In this specification, "exogenous glycoproteins" refers to glycoproteins whose expression in plants is the result of genetic engineering methodologies. Examples of these 25 exogenous glycoproteins include enzymes, hormones, cytokines, antibodies, vaccines, receptors and serum proteins. Examples of enzymes include horseradish peroxidase, kinase, glucocerebrosidase,  $\alpha$ -galactosidase, tissue-type plasminogen activator (TPA), and HMG-CoA 30 reductase. Examples of hormones and cytokines include enkephalin, interferon alpha, GM-CSF, G-CSF, chorion stimulating hormone, interleukin-2, interferon-beta, interferon-gamma, erythropoietin, vascular endothelial

growth factor, human choriogonadotropin (HCG),  
leuteinizing hormone (LH), thyroid stimulating hormone  
(TSH), prolactin, and ovary stimulating hormone. Examples  
of antibodies include IgG and scFv. Examples of vaccines  
5 include antigens such as Hepatitis B surface antigen,  
rotavirus antigen, Escherichia coli enterotoxin, malaria  
antigen, rabies virus G protein, and HIV virus glycoprotein  
(e.g., gp120). Examples of receptors and matrix proteins  
include EGF receptors, fibronectin,  $\alpha$ 1-antitrypsin, and  
10 coagulation factor VIII. Examples of serum proteins  
include albumin, complement proteins, plasminogen,  
corticosteroid-binding globulin, throxine-binding  
globulin, and protein C.

15 In this specification, "genes of exogeneous glycoproteins"  
refers to a gene, which can be isolated from a cell using  
a nucleotide sequence of an encoded protein well known in  
the art, or commercially available genes altered for  
expression in plants.

20 The gene of the enzymes capable of conducting a transfer  
reaction of a galactose residue to a non-reducing terminal  
acetylglucosamine residue and the genes of exogenous  
glycoproteins can be introduced to the plant cells using  
25 a method well known in the art. These genes can be introduced  
separately or simultaneously. Examples of methods for  
introducing genes to plant cells include the Agrobacterium  
method, the electroporation method and the particle  
bombardment method.

30 Using any method well known in the art, the plant cells with  
introduced genes can be tested to make sure the introduced  
genes are expressed. Examples of such methods include

silver staining or augmentation, Western blotting, Northern hybridization, and enzyme activity detection. Cells that express the introduced genes are referred to as transformed cells.

5 Transformed cells, which express enzymes capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and exogenous glycoproteins, express exogenous glycoproteins with human-type sugar chains. In other words, the  
10 transformed cells have human-type sugar chain adding mechanisms. By cultivating these transformed cells, glycoproteins with human-type sugar chains can be mass produced. Human-type glycoproteins contain core sugar chains and outside sugar chains. The core sugar chains  
15 consist essentially of a plurality of mannose or acetylglucosamine. The outside sugar chains in these glycoproteins contain non-reducing terminal sugar chain portions. The outside sugar chains can have a straight chain configuration or a branched chain configuration. The  
20 branched sugar chain portion has a mono-, bi-, tri- or tetra configuration. The glycoproteins manufactured using these transformed cells ideally do not contain any fucose or xylose.

25 These transformed plant cells can remain in a cultured state or can be differentiated into specific tissues or organs. Alternatively, they can also be generated into plants. In this case, the transformed plant cells can be present in the entire plant or in specific portions of the plant, such  
30 as seed, fruit, nut, leaf, root, stem or flower of the plant.

Glycoproteins with human-type sugar chains can be manufactured by the transformed plant cells and then be

isolated or extracted from the plant cells. The method for isolating the glycoproteins can be any method well known in the art. The glycoproteins of the present invention can be used in foodstuffs while remaining inside the transformed  
5 cells, or the glycoproteins of the present invention can be administered to animals including humans without antigenicity because of the added human-type sugar chains.

Hereinafter, the present invention will be described in  
10 detail by way of illustrative, but not restrictive, examples.

(Example 1) Cloning Human  $\beta$ 1,4 Galactose Transferase Genes  
 $\beta$ 1,4 Galactosyltransferase (hGT) genes (EC2.4.1.38) have  
15 already been cloned. A primary configuration consisting of 400 amino acids has been discovered (Masri, K.A. et al., Biochem. Biophys. Res. Commun., 157, 657-663, 1988).

(1) Primer Preparation and Template DNA

The following primers were prepared with reference to the  
20 report by Masri et al.

hGT-5Eco: 5'-AAAGAATTTCGCGATGCCAGGCGCGTCCCT-3' (Sequence ID:1)

hGT-2Sal: 3'-TCGATCGCAAAACCATGTGCAGCTGATG-5' (Sequence I.D:2)

25 hGT-7Spe: 3'-ACGGGACTCCTCAGGGGCGATGATCATAA-5' (Sequence I.D:3)

hGT6Spe: 5'-AAGACTAGTGGGCCCCATGCTGATTGA-3' (Sequence I.D:4)

Human genome DNA, human placenta cDNA, and human kidney cDNA  
30 purchased from Clontech were used as the template DNA.

(2) Cloning the hGT Gene cDNA

(i) Human genome DNA was used as the template and hGT-5Eco

and hGT-7Spe were used as the primers; (ii) Human placenta cDNA was used as the template and hGT-2Sal and hGT6Spe were used as the primers. The two were combined and a PCR reaction was performed under the following conditions. Then, 0.4 kb and 0.8 kb fragments containing hGT encoded areas were obtained.

(PCR reaction mixture) 1  $\mu$ l template DNA, 5  $\mu$ ml 10 x PCR buffer solution, 4  $\mu$ l dNTPs (200 mM), the primers (10 pmol), and 0.5  $\mu$ l (Takara Shuzo Co., Ltd.) Tag polymerase (or 0.2  $\mu$ l Tub polymerase), water was added to make 50  $\mu$ l.

(PCR Reaction Conditions) First Stage: 1 cycle, denaturation (94°C) 5 minutes, annealing (55°C) 1 minute, extension (72°C) 2 minutes. Second Stage: 30 cycles, denaturation (94°C) 1 minute, annealing (55°C) 1 minute, extension (72°C) 2 minutes. Third Stage: 1 cycle, denaturation (94°C) 1 minute, annealing (55°C) 2 minutes, extension (72°C) 5 minutes.

The two fragments were combined to form hGT gene cDNA and cloned in pBluescript II SK+ (SK). The pBluescript II SK+ (SK) was purchased from Stratagene Co., Ltd. FIG 2 shows the structure of a plasmid containing hGT gene cDNA. This shows Sequence No. 5 in the hGT gene nucleotide sequence and Sequence No. 6 in the estimated amino acid sequence. This nucleotide sequence differed from the hGT sequence published by Masri et al. (see above) in the following ways:

a) The nucleotides are different in that the A in Position No. 528 is G, the C in Position No. 562 is T, and the A in Position No. 1047 is G, however the encoded amino acid sequence is not changed; b) Nine nucleotides at positions from Position No. 622 to Position No. 630 are missing; c) The G in Position No. 405 is A and the T in Position No. 408 is A. These nucleotide changes were made during primer preparation such that the 0.4 kb fragment and 0.8 kb fragment

are connected. There are two start codons (ATG) in hGT gene cDNA. In this experiment, however, the gene is designed such that translation begins from the second initial codon (Position No. 37).

5

(Example 2) Introduction of the hGT Gene to a Cultivated Tobacco Cell

(1) It has been reported that hGT is expressed in an active form in *Escherichia coli* (Aoki, D. et al., EMBO J., 9, 3171, 1990 and Nakazawa, K. et al., J. Biochem., 113, 747, 1993). In order for a cultivated tobacco cell to express hGT, the expression vector pGAhGT had to be structured as shown in FIG 3. A cauliflower mosaic virus 35S promoter (CaMV 35S promoter), which drives gene expression constitutively in plant cells, was used as the promoter. A kanamycin-resistance gene was used as the selection marker. The pGAhGT was introduced to the cultivated tobacco cell by means of *Agrobacterium* method.

20 The *Agrobacterium* method was performed using the triparental mating method of Bevan et al. (Bevan, M., Nucleic Acid Res., 12, 8711, 1984). *Escherichia coli* DH5 $\alpha$  (suE44, DlacU169, ( $\phi$ 80lacZDM15), hsdR17) (Bethesda Research Laboratories Inc.: Focus 8 (2), 9, 1986) with pGA-type plasmids (An. G., 25 Methods Enzymol. 153, 292, 1987) and *Escherichia coli* HB101 with helper plasmid pRK2013 (Bevan, M., Nucleic Acid Res., 12, 8711, 1984) were left standing overnight and 37°C in a 2 x YT medium containing 12.5 mg/l tetracycline and 50 mg/l kanamycin, and *Agrobacterium tumefaciens* EHA101 was left 30 standing over two nights at 28°C in a 2 x YT medium containing 50 mg/l kanamycin and 25 mg/l chloramphenicol. Then, 1.5 ml of each cultured medium was removed and placed into an Eppendorf tube. After the cells of each strain were

collected, the cells were rinsed three times in an LB medium. The cells obtained in this manner were then suspended in 100  $\mu$ l of a 2 x YT medium, mixed with three types of bacteria, applied to a 2 x YT agar medium, and cultivated at 28°C whereby the pGA-type plasmids, then underwent conjugal transfer from the *E. coli* to the *Agrobacterium*. Two days later some of the colony appearing on the 2 x YT agar plate was removed using a platinum loop, and applied to an LB agar plate containing 50 mg/l kanamycin, 12.5 mg/l tetracycline, and 25 mg/l chloramphenicol. After cultivating the contents for two days at 28°C, a single colony was selected.

Transformation of the cultivated tobacco cells was performed using the method described in An, G., Plant Mol. Bio. Manual, A3, 1. First, 100  $\mu$ l of *Agrobacterium* EHA101 with pGA-type plasmids cultivated for 36 hours at 28°C in an LB medium containing 12.5 mg/l tetracycline and 4 ml of a suspension of cultivated tobacco cells *Nicotiana tabacum* L. cv. bright yellow 2 (Strain No. BY-2 obtained using Catalog No. RPC1 from the Plant Cell Development Group of the Gene Bank at the Life Science Tsukuba Research Center), in their fourth day of cultivation, were mixed together thoroughly in a dish and allowed to stand in a dark place at 25°C. Two days later, some of the solution was removed from the dish and the supernatant was separated out using a centrifuge (1000 rpm, 5 minutes). The cell pellet was introduced to a new medium and centrifuged again. The cells were innoculated onto a modified LS agar plate with 150-200 mg/l kanamycin and 250 mg/l carbenicillin. This was allowed to stand in darkness at 25°C. After two to three weeks, the cells grown to the callus stage were transferred to a new plate and clones were selected. After two to three weeks, the clones were transferred to a 30 ml modified LS medium with kanamycin

and carbenicillin. This selection process was repeated over about one month. Six resistant strains were randomly selected from the resistant strains obtained in this manner (GT 1, 4, 5, 6, 8 and 9).

5

#### (2) Verification of the Introduced hGT Genes

In the resistant strains, a 2.2 kb fragment containing a CaMV35S promoter and an hGT gene cDNA-NOS terminator in the T-DNA was confirmed in the genomic DNA of the cultivated tobacco cells using a Southern blot analysis. The Southern method was performed after the genomic DNA had been prepared from the resistant strains mentioned above and digested by EcoRI and HindIII.

15 The preparation of the chromosomal DNA from the cultured tobacco cells was performed using the Watanabe method (Watanabe, K., Cloning and Sequence, Plant Biotechnology Experiment Manual, Nouseon Bunka Co., Ltd.). First, 10 ml of the cultivated tobacco cells were frozen using liquid  
20 nitrogen, and then ground to powder using a mortar and pestle. About five grams of the powder was placed in a centrifuge tube (40 ml) rapidly such that the frozen powder did not melt and mixed with 5 ml of a 2 x CTAB (cetyltrimethyl ammonium bromide) solution pre-heated to 60°C. This was well mixed,  
25 slowly for 10 minutes, and then allowed to stand at 60°C. Then, 5 ml of a chloroform:isoamylalcohol (24:1) solution was added, and the mixture was stirred into an emulsion. The mixture was then centrifuged (2,800 rpm, 15 minutes, room temperature). The surface layer was then transferred  
30 to a new 40 ml centrifuge tube and the extraction process was repeated using the chloroform:isoamylalcohol (24:1) solution. After the surface layer had been mixed with 1/10 volume of 10% CTAB, it was centrifuged (2,800 rpm, 15 minutes,



room temperature). The surface layer was transferred to a new centrifuge tube and then mixed with an equal volume of cold isopropanol. The thus obtained solvent mixture was then centrifuged (4,500 rpm, 20 minutes, room temperature).  
5 After the supernatant had been removed using an aspirator, it was added to 5 ml of a TE buffer solution containing 1 M sodium chloride. This was completely dissolved at 55-60°C. This was mixed thoroughly with 5 ml of frozen isopropanol and the DNA was observed. It was placed on the  
10 tip of a chip, transferred to an Eppendorf tube (containing 80% frozen ethanol), and then rinsed. The DNA was then rinsed in 70% ethanol and dried. The dried pellet was dissolved in the appropriate amount of TE buffer solution. Then, 5 ml of RNAase A (10 mg/ml) was added, and reacted  
15 for one hour at 37°C; Composition of the 2 x CTAB Solution: 2% CTAB, 0.1 M Tris-HCl (pH8.0), 1.4 M sodium chloride, 1% polyvinylpyrrolidone (PVP); composition of the 10% CTAB solution: 10% CTAB, 0.7 M sodium chloride.

20 The Southern blot method was performed in the following manner:

(i) DNA Electrophoresis and Alkali Denaturation: After 40  $\mu$ g of the chromosomal DNA had been completely digested by the restriction enzyme, the standard method was used, and  
25 1.5% agarose gel electrophoresis was performed (50 V). It was then stained with ethidium bromide and photographed. The gel was then shaken for 20 minutes in 400 ml of 0.25 M HCl, and the liquid removed, and the gel permeated with 400 ml of a denaturing solution (1.5 M NaCl, 0.5 M NaOH by  
30 shaking slowly for 45 minutes. Next, the liquid was removed, 400 ml of neutral solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) was added, and the solution was shaken slowly for 15 minutes. Then, 400 ml of the neutral solution was again

added, and the solution was shaken slowly again for 15 minutes. (ii) Transfer: After electrophoresis, the DNA was transferred to a nylon membrane (Hybond-N Amersham) using 20 x SSC. The transfer took more than 12 hours. After the

5 blotted membrane was allowed to dry at room temperature for an hour, UV fixing was performed for five minutes. 20 x SSC Composition: 3 M NaCl, 0.3 M sodium citrate. (iii) DNA Probe Preparation: The DNA probe preparation was performed using a Random Prime Labeling Kit (Takara Shuzo Co., Ltd.). Next,

10 the reaction solution was prepared in an Eppendorf tube. After the tube was heated for three minutes to 95°C, it was rapidly cooled in ice. Then, 25 ng of the template DNA and 2  $\mu$ l of the Random Primer were added to make 5  $\mu$ l. Then, 2.5  $\mu$ l 10 x buffer solution, 2.5  $\mu$ ml dNTPs, and 5  $\mu$ l [ $\alpha$ -

15  $^{32}$ P] dCTP (1.85 MBq, 50 mCi) were added, and H<sub>2</sub>O was added to bring the volume of reaction mixture to 24  $\mu$ l. Then, 1  $\mu$ l of a Klenow fragment was added and the solution was allowed to stand for 10 minutes at 37°C. It was then passed through a NAP10 column (Pharmacia Co., Ltd.) to prepare the

20 purified DNA. After being heated for three minutes at 95°C, it was rapidly cooled in ice, and used as a hybridization probe. (iv) Hybridization: 0.5% (w/v) SDS was added to the following Pre-hybridization Solution, the membrane in (ii) was immersed in the solution, and pre-hybridization was

25 performed for more than two hours at 42°C. Afterwards, the DNA probe prepared in (iii) was added, and hybridization was performed for more than 12 hours at 42°C. Composition of the Pre-hybridization Solution: 5 x SSC, 50 mM sodium phosphate, 50% (w/v) formamide, 5 x Denhardt's solution

30 (prepared by diluting 100 x Denhardt's solution), 0.1% (w/v) SDS. Composition of the 100 x Denhardt's Solution: 2% (w/v) BSA, 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone (PVP). (v) Autoradiography: After rinsing in the manner described

below, autoradiography was performed using the standard method. It was performed twice for 15 minutes at 65°C in 2 x SSC and 0.1% SDS, and once for 15 minutes at 65°C in 0.1 x SSC and 0.1% SDS.

5

The results of the Southern blot analysis of the genome DNA prepared from the resistant strains are shown in FIG 4. As shown in FIG 4, the presence of the hGT gene was verified in four strains (GT1, 6, 8 and 9).

10

(Example 3). Analysis of the Galactosyltransferase Transformant

The cells of the transformants (GT-1, 6, 8 and 9) and wild-type BY-2 in the fifth through seventh day's culture both were harvested, and then suspended in extraction buffer solution (25 mM Tris-HCl, pH 7.4; 0.25 M sucrose, 1 mM MgCl<sub>2</sub>, 50 mM KCl). The cells were ruptured using ultrasound processing (200 W; Kaijo Denki Co., Ltd. Japan) or homogenized. The cell extract solution and the microsome fractions were then prepared according to the method of Schwientek, T. et al. (Schwientek, T. and Ernst, J.F., Gene 145, 299-303, 1994). The expression of the hGT proteins was detected using Western blotting and anti-human galactosyltransferase (GT) monoclonal antibodies (MAb 8628; 1:5000) (Uejima, T. et al., Cancer Res., 52, 6158-6163, 1992; Uemura, M. et al., Cancer Res., 52, 6153-6157, 1992) (provided by Professor Narimatsu Hisashi of Soka University). Next, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (5% skim milk 1:1000; EY Laboratories, Inc., CA), and a colorimetric reaction using horseradish peroxidase was performed using the POD Immunoblotting Kit (Wako Chemicals, Osaka).

An immunoblot analysis of the complex glycans unique to plants was performed using polyclonal antiserum against  $\beta$ -fructosidase in the cell walls of carrots and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (5% skim milk 1:1000; Sigma) (Lauriere, M. et al., Plant Physiol. 90, 1182-1188, 1989).

The  $\beta$ 1,4-galactosyltransferase activity was assayed as a substrate using UDP-galactose and a pyridylamino (PA-) labeled GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> (GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA) (Morita, N. et al., J. Biochem. 103, 332-335, 1988). The enzyme reaction solution contained 1-120  $\mu$ g protein, 25 mM sodium cacodylate (pH 7.4), 10 mM MnCl<sub>2</sub>, 200 mM UDP-galactose, and 100 nM GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA. An HPLC analysis was performed on the reaction product using PALPAK Type R and PALPAK Type N columns (Takara Shuzo Co., Ltd.) and the method recommended by the manufacturer. The GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA used as the standard marker was used along with Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA and two isomers of GalGlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA purchased from Takara Shuzo Co. Ltd. and Honen Co., Ltd.

The immunoblottings for the proteins derived from the transformant and the wild-type cells are shown in FIG 5. As shown in FIG 5, positive signals of a molecular weight of 50 kDa were observed. This is greater than the molecular weight estimated from the amino acid sequence (40 kDa) and is roughly equivalent to the bovine galactosyltransferase purified from ascites and expressed in yeast (Uemura, M. et al., Cancer Res., 52, 6153-6157, 1992; Schwientek, T. et al., J. Biol. Chem., 271 (7), 3398-3405, 1996). In the microsome fraction, immunoreactive bands (FIG 5, Lanes 1,4) stronger than those of the cell lysate (FIG 5, Lanes 6-8)

were observed. This means that hGT is localized preferentially in the cell. No immunoreactive bands were detected in the wild-type cells.

5 The proteins in the microsome fractions of transformant GT6 and wild-type BY-2 were bound in an RCA<sub>120</sub> agarose column (Wako Chemicals, Osaka), and then rinsed with 15 volumes of 10 mM ammonium acetate pH 6.0. Next, the bound proteins were  
10 eluted using 0.2 M lactose. After separation using SDS-PAGE, the proteins were stained using silver staining (Wako Silver Staining Kit) (FIG 6) or lectin (FIG 7). In the lectin staining, the membrane blots were rinsed in a TTBS buffer solution (10 mM Tris-HCl, pH 7.4; 0.15 M NaCl; 0.05% Tween 20) and incubated with horseradish peroxidase labeled RCA<sub>120</sub>  
15 (Honen Co., Ltd.). Galactosylated glycan was then observed using a Immunoblotting Kit (Wako Chemicals, Osaka) (FIG 7). As shown in FIG 7, an RCA<sub>120</sub> binding was not observed in the wild-type BY2 cells, and the GT6 had a glycoprotein with galactose on the non-reducing terminus of the glycan  
20 portion.

The protein extract from the wild-type BY2 cells and the GT6 cells as well as the GT6 proteins eluted from the RCA<sub>120</sub> affinity chromatography were probed using polyclonal  
25 antibodies unique to complex glycan (FIG 8). The antiserum binds predominantly to the  $\beta$ 1,2-xylose residue on the plant glycoprotein (Lauriere, M. et al., Plant Physiol. 90, 1182-1188, 1989). As shown in FIG 8, the wild-type BY2 cells (Lane 1) contain glycoproteins that reacted with the  
30 polyclonal antiserum. GT6 contains very few glycoproteins that reacted with the polyclonal antiserum (Lane 2). The GT6 glycoproteins eluted from RCA<sub>120</sub> affinity chromatography did not bind to the polyclonal antiserum, indicating that

the galactosylated glycan does not contain  $\beta$  1,2-xylose residue (Lane 3).

(Example 4) Introduction of the Horseradish Peroxidase (HRP) gene to the hGT-Introduced Cultivated Tobacco Cells

Horseradish peroxidase gene was introduced to the resultant GT6 cell line. Among the different types of plant peroxidase, horseradish peroxidase, especially HRP isozyme C, HRP (EC1.11.1.7) has been the subject of extensive research.

HRP can be used in various enzyme reactions because of its superior stability and a broad spectrum of substance specificity. For example, it has been used in enzyme immunology for binding with a secondary antibody in Western blotting. A number of horseradish peroxidase isozyme genes have now been cloned (Fujiyama, K. et al., Eur. J. Biochem., 173, 681-687, 1988 and Fujiyama, K. et al., Gene, 89, 163-169, 1990). ClaPeroxidase (ClaPRX) which is encoded by prxCla is first translated as a protein consisting of 353 amino acids containing an extra peptide consisting of 30 amino acids at the N terminus and 15 amino acids at the C terminus. Then, this is processed to form a mature enzyme with 308 amino acids (Fujiyama, K. et al., Eur. J. Biochem., 173, 681-687, 1988). The molecular weight of ClaPRX ranges between 42,200 and 44,000. Of this molecular weight, sugar chains account for 22-27%, and there are eight N-linked sugar chains (Welinder, K.G., Eur. J. Biochem., 96, 483-502, 1979). The introduction of the ClaPRX gene was performed using the binary vector pBIHm-HRP for HRP expression shown in FIG 9.

The pBIHm-HRP was prepared in the following manner. First, a 1.9 kbp HindIII-SacI fragment was prepared from a vector 35S-prxCla for plant expression, which carries an HRP cDNA (Kawaoka, A. et al., J. Ferment. Bioeng., 78, 49-53, 1994).

The HindIII-SacI fragment contains a full length 1.1 kbp prxCla cDNA following a 0.8 kbp CaMV35S promoter. The 1.9 kbp HindIII-SacI fragment was inserted in the HindIII-SacI site of the binary vector pBI101HmB (Akama, K. et al., Plant Cell Rep., 12, 7-11, 1992). The BamHI site at 3' of the hygromycin resistant gene (HPT gene) had been destroyed.

Because the GT6 strain is kanamycin resistant, the hygromycin-resistant hpt gene was used as the selection marker (Griz, L. and Davies J., Gene, 25, 179-188, 1983). The transformation of the GT6 strain by HRP gene was performed using the method described in Rempel, D.H. and Nelson, L.M. (Rempel, D.H. and Nelson, L.M., Transgenic Res. 4: 199-207, 1995). In order to obtain HRP transformant as a control, an HRP gene was introduced to a wild-type BY2 cell to obtain a BY2-HRP strain. The double-transformant GT6-HRP with hGT and HRP was obtained in which an ordinary transformation process takes place.

(Example 5) Verification of the Expression of HRP in the Cultivated Double-Transformant Tobacco Cells

Double transformant GT6-HRP, control BY2-HRP and wild-type (WT) cell line were examined for the expression of HRP activity using the following method. As seen in Table 1, the HRP gene-introduced transformant had peroxidase activity about five times higher than the wild-type cell line.

Table 1

Clone Number	Specific activity [U/mg protein]
WT-HRP-1	10.3
WT-HRP-5	11.3
WT-HRP-7	12.6

GT-HRP-4	11.1
GT-HRP-5	9.35
GT-HRP-8	9.47
Wild Type	2.49

Clone BY2-HRP obtained by introducing the HRP gene to the wild type expressed the same degree of peroxidase activity as the GT6-HRP double transformant with hGT and HRP.

5

(Peroxidase Activity Measurement)

The cultivated tobacco cells were placed into an Eppendorf tube containing Solution D and were ruptured using a homogenizer (Homogenizer S-203, Ikeda Rika Co., Ltd.). The supernatant was collected after centrifugation (12,000 rpm, 20 minutes, 4°C) and then used as the crude enzyme solution. Next, 1 ml of Solution A, 1 ml of Solution B and 2 ml of Solution C were mixed together, and the mixture was incubated for five minutes at 25°C. The crude enzyme solution appropriately diluted with Solution D was added to the mixture, and allowed to react for three minutes at 25°C. The reaction was stopped by the addition of 0.5 ml of 1 N HCl, and the absorbance at 480 nm was measured. As a control, a solution with 1 N HCl added before the introduction of the enzyme was used.

20

Solution A: 1 mM o-aminophenol

Solution B: 4 mM H<sub>2</sub>O<sub>2</sub>

Solution C: 200 mM sodium phosphate buffer (pH 7.0)

25

Solution D: 10 mM sodium phosphate buffer (pH 6.0)

30

Next, in order to determine whether or not the rise in peroxidase activity was due to the expression of HRP, activity staining was performed after separation by gel isoelectric focusing. The isoelectric focusing was



performed using a BIO-RAD Model 111 Mini-IEF Cell. The hydrophobic surface of the PAGE gel support film was attached to a glass plate, and then placed on a casting tray. The prepared gel solution was poured between the support film and the casting tray and then photopolymerized for 45 minutes under a fluorescent lamp. The sample was applied to the gel, and the gel was positioned so as to come into contact with both graphite electrodes wetted with distilled water in the electrophoretic bath. Electrophoresis was then performed for 15 minutes at 100 V, 15 minutes at 200 V and 60 minutes at 450 V. Composition of the Gel Solution (per 1 Gel Sheet): distilled water 2.75 ml, acrylamide (25%T, 3%C) 1.0 ml, 25% glycerol 1.0 ml, Bio-lite (40%, pH 3-10) 0.25 ml, 10% ammonium persulfate 7.5  $\mu$ l, 0.1% sodium riboflavin5'-phosphate 25  $\mu$ l, TEMED 1.5  $\mu$ l.

The activity staining of peroxidase was performed according to the method of Sekine et al. (Sekine et al., Plant Cell Technology, 6, 71-75, 1994). As shown in FIG 10, a significant band not found in wild-type cell line was detected in the pI 7.8 position in the BY2-HRP cell line and the GT6-HRP strain. The results of a Western analysis using anti-HRP antibodies confirmed the detection of a signal at the position corresponding to pI 7.8 and the expression of HRP in the double transformant GT6-HRP with hGT and HRP.

#### (Example 6) Structural Analysis of the N-linked Sugar Chains in the Transformant GT6 Cells

##### 30 (Method Used to Analyze the Sugar Chain Structure)

The N-linked sugar chains in the transformant GT6 cells were analyzed by combining reverse-phase HPLC and size-fractionation HPLC, performing the two-dimensional PA sugar

chain mapping, performing exoglycosidase digestion, and then performing ion spray tandem mass spectrometry (IS-MS/MS) (Perkin Elmer Co., Ltd.). First, the cell extract solution was delipidated with acetone, treated with hydrazine for 12 hours at 100°C, and the sugar chain portion was released. The hydrazinolysate was N-acetylated, desalted using the Dowex 50X2 and the Dowex 1X2 (The Dow Chemical Co., Ltd. and its representative in Japan, Muromachi Chemical Industry Co., Ltd.), then fractionized by using 0.1 N ammonia and the Sephadex G-25 gel filtration column (1.8 x 180 cm) (Pharmacia Co., Ltd.). Pyridylamination was then performed as described above. The pyridylaminated sugar chains (PA sugar chains) were then separated using a Jasco 880-PU HPLC device with a Jasco 821-FP Intelligent Spectrophotometer (Japan Spectroscopic Co., Ltd.) and Cosmosil 5C18-P and Asahipak NH2P-50 columns. The elution positions were compared with a standard either produced by the applicant or purchased (from Wako Pure Chemical Industries, Ltd. and Takara Shuzo Co., Ltd.).

The glycosidase digestion using N-acetyl- $\beta$ -D-glucosaminidase (*Diplococcus pneumoniae*, Boehringer Mannheim) or mannosidases (Jack bean, Sigma) was performed on about 1 nmol of the PA sugar chains under the same conditions as the method described in Kimura, Y. et al., Biosci. Biotech. Biochem. 56 (2), 215-222, 1992. Digestion using  $\beta$ -galactosidase (*Diplococcus pneumoniae*, Boehringer Mannheim) or *Aspergillus saitoi*-derived  $\alpha$ -1,2 mannosidase (provided by Dr. Takashi Yoshida at Tohoku University) was performed by adding 1 nmol of PA sugar chains and 200 mU  $\beta$ -galactosidase or 60  $\mu$ g of  $\alpha$ -1,2 mannosidase to 50 mM of sodium acetate buffer (pH 5.5) and incubating at 37°C. After the resultant reaction solution was boiled and the enzyme

reaction was stopped, a portion of the digested product was analyzed using size-fractionation HPLC. The molecular weight of the digested product was analyzed using ion spray tandem mass spectrometry (IS-MS/MS) and/or compared to the standard sugar chain as described in Palacpac, N.Q. et al., Biosci. Biotech. Biochem. 63(1) 35-39, 1999 and Kimura, Y. et al., Biosci. Biotech. Biochem. 56 (2), 215-222, 1992.

The IS-MS/MS experiment was performed using a Perkin Elmer Sciex API-III. It was performed in positive mode with an ion spray voltage of 4200 V. Scanning was performed every 0.5 Da, and the m/z was recorded from 200.

(Analysis of the Sugar Chains in the GT6 Cells)

The PA sugar chains prepared from the GT6 cells were purified and analyzed using a combination of reverse-phase HPLC and size-fractionation HPLC. In Fraction I at the 10-20 minute positions in the size-fractionation HPLC (FIG 11), no N-linked sugar chains were eluted. This suggests that the Fraction I is a non-absorption portion containing by-products of hydrazinolysis. In the MS/MS analysis, no fragment ion with m/z values of 300, which corresponds to PA-GlcNAc, was detected. Similarly, Fraction XI at the 50-60 minute positions did not have a peak indicating elution by the size-fractionation HPLC. Therefore, it is clear that there were no N-linked sugar chains. The 17 peaks including A-Q shown in FIG 12 were all collected and purified after the analysis from Fraction II to Fraction X in the size-fractionation HPLC (FIG 11) was completed.

30

The IS-MS/MS analysis found that seven of these peaks were N-linked sugar chains. The following is the result from the analysis of these peaks.

The elution positions and molecular weights of oligosaccharides -A, -E, -H, -I, -M, -O, -P and -Q (FIG 12) did not correspond to those of PA sugar chain standards.

5 In the MS/MS analysis, the  $m/z$  values of 300 and 503, which respectively correspond to PA-GlcNAc and PA-GlcNAc<sub>2</sub>, were detected. However, the fragment ions were not detected corresponding to ManGlcNA<sub>2</sub> (M1) or the trimannose core sugar chain Man<sub>3</sub>GlcNAc<sub>2</sub> (M3) which are generally found in N-linked

10 sugar chain (data not shown). Even the oligosaccharides -B, -D and -N at the other peaks did not have fragment ions detected with an  $m/z$  value of 300. Thus, these were not N-linked sugar chains. The seven remaining N-linked sugar chains were then examined.

15

The elution positions and molecular weights of peak-C ( $m/z$  1637.5; molar ratio 9.3%), peak-F ([M+2H]<sup>2+</sup> +  $m/z$  819.5, [M+H]<sup>+</sup> +  $m/z$  1639; molar ratio 15.9%), and peak-G ( $m/z$  1475.5; molar ratio 19.5%) indicated high mannose-type sugar chains

20 Man<sub>7</sub>GlcNAc<sub>2</sub> (Isomer M7A and M7B) and Man<sub>6</sub>GlcNAc<sub>2</sub> (M6B) respectively. When digested by Jack bean  $\alpha$ -mannosidase, it was indicated that the N-linked sugar chains are degraded to ManGlcNAc (M1) by size-fractionation HPLC analysis (data not shown). In an IS-MS experiment on the digestion product,

25 the ion with an  $m/z$  value of 665.5 corresponding to a calculated value of 664.66 for M1 was detected, indicating that these N-linked sugar chains have the same structure as respective corresponding PA sugar chain standard.

30

Peak-J (6.6%) had a molecular weight of 1121.5, which is almost the same as the calculated molecular weight value of  $m/z$  1121.05 of Man<sub>3</sub>Xyl<sub>1</sub>GlcNAc<sub>2</sub>-PA (M3X). The positions of the fragment ions were 989.5, 827.5, 665.5, 503.3 and

300. This does not contradict the findings that Xyl, Man, Man, Man, and GlcNAc were released in successive order from Man<sub>3</sub>Xyl<sub>1</sub>GlcNAc<sub>2</sub>-PA. When digested using Jack bean  $\alpha$ -mannosidase, the mannose residues on the non-reducing terminus can be removed, and the two-dimensional mapping revealed the same elution positions as those of Man<sub>1</sub>Xyl<sub>1</sub>GlcNAc<sub>2</sub>-PA (data not shown).

The results of the analysis of the IS-MS experiment on peak-K (13.2%) fraction revealed that this fraction contains two types of N-linked sugar chains, one has the molecular weight of 1314.0 (1.4%) and the other has the molecular weight of 1354.5 (11.8%). This fraction was subjected to reverse-phase HPLC, purified and analyzed. The sugar chain peak K-1 with a molecular weight of 1314.0 had the same two-dimensional mapping and m/z value measured as that of the sugar chain standard Man<sub>5</sub>GlcNAc<sub>2</sub>-PA (M5). When treated using jack bean  $\alpha$ -mannosidase, the elution positions of the degraded product had shifted to positions similar to those of M1 in the two-dimensional mapping. This indicates the removal of four mannose residues.

(Galactose-added N-linked Type Sugar Chains in the GT6 Cells)

The determined m/z value of 1354.5 for sugar chain peak K-2 is almost the same as the molecular weight m/z value of 1354.3 predicted for Gal<sub>1</sub>GlcNAc<sub>1</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA (GalGNM3). The result of the mass spectrometry indicated that fragment ions were derived from the parent molecules. The m/z value of 1193.5 indicated GlcNAc<sub>1</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 989.5 indicated Man<sub>3</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 827.5 indicated Man<sub>2</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 665 indicated ManGlcNAc<sub>2</sub>-PA, the m/z value of 503 indicated GlcNAc<sub>2</sub>-PA,

the m/z value of 336 indicated ManGlcNAc, the m/z value of 300 indicated GlcNAc-PA, and the m/z value of 204 indicated GlcNAc. From the putative N-linked sugar chain structure, it is considered to be either of two GalGNM3 isomers (FIG 13). It is either Gal  $\beta$  4GlcNAc  $\beta$  2Man  $\alpha$  6(Man  $\alpha$  3)Man  $\beta$  4GlcNAc  $\beta$  4GlcNAc-PA or Man  $\alpha$  6(Gal  $\beta$  4GlcNAc  $\beta$  2Man  $\alpha$  3)Man  $\beta$  4GlcNAc  $\beta$  4GlcNAc-PA. The purified PA sugar chains had reverse-phase HPLC elution positions that were the same as the sugar chain standard Man  $\alpha$  6(Gal  $\beta$  4GlcNAc  $\beta$  2Man  $\alpha$  3) Man  $\beta$  4GlcNAc  $\beta$  4GlcNAc-PA (FIG 13B).

The sugar chain was treated with exoglycosidase and the structure of the sugar chain was verified. The *D. pneumoniae*  $\beta$ -galactosidase is a Gal  $\beta$  1,4GlcNAc linkage specific enzyme. The digested product of the sugar chain by the enzyme was eluted at the same position as that of the GlcNAc<sub>1</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-PA in the size-fractionation HPLC (FIG 14A-II). An m/z of 1192.0 was obtained from the IS-MS/MS analysis. These results indicate a galactose residue has been removed from the GlcNAc on the non-reducing terminus with the  $\beta$  1,4 binding. When the product was digested by a N-acetyl- $\beta$ -D-glucosaminidase derived from *Diplococcus pneumoniae*, which is  $\beta$  1,2 GlcNAc linkage specific (Yamashita, K. et al., J. Biochem. 93, 135-147, 1983), the digested product was eluted at the same position as that of the standard Man<sub>3</sub>GlcNAc<sub>2</sub>-PA in the size-fractionation HPLC (FIG 14A-III). When the digested product was treated with jack bean  $\alpha$ -mannosidase, it was eluted at the same position as that of the standard ManGlcNAc<sub>2</sub>-PA in the size-fractionation HPLC (FIG 14A-IV). The sugar chain structure is shown in K-2 of FIG 15.

The mass spectroscopy analysis of Peak L(35.5%) gave [M+2H]

2+ of 840, [M+H]<sup>+</sup> of 1680.0, which nearly matched the molecular weight m/z value of 1678.55 expected for Gal<sub>1</sub>GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>-PA (GalGNM5). The result of the mass spectrometry indicated fragment ions derived from the parent molecules. The m/z value of 1313.5 indicated Man<sub>5</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 1152 indicated Man<sub>4</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 989.5 indicated Man<sub>3</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 827.5 indicated Man<sub>2</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 665 indicated ManGlcNAc<sub>2</sub>-PA, the m/z value of 503 indicated GlcNAc<sub>2</sub>-PA, the m/z value of 336 indicated ManGlcNAc, the m/z value of 300 indicated GlcNAc-PA, and the m/z value of 204 indicated GlcNAc. The product digested with *D. pneumoniae*  $\beta$ -galactosidase was eluted at the same position as that of GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>-PA in the size-fractionation HPLC (FIG 14B-II). The results indicate that a galactose residue is bound to the GlcNAc on the non-reducing terminus with the  $\beta$ 1,4 linkage. The removal of the galactose was confirmed by the molecular weights obtained from the IS-MS/MS analysis. [M+2H]<sup>2+</sup> was 759 and [M+H]<sup>+</sup> was 1518.0. The mass spectrometry indicated fragments ions derived from the GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>-PA with a parent signal of m/z 1518.0. The m/z value of 1314 indicated Man<sub>5</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 1152 indicated Man<sub>4</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 990 indicated Man<sub>3</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 827.5 indicated Man<sub>2</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 665.5 indicated Man<sub>1</sub>GlcNAc<sub>2</sub>-PA, the m/z value of 503 indicated GlcNAc<sub>2</sub>-PA, and the m/z value of 300 indicated GlcNAc-PA. When the GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>-PA was digested with an N-acetyl- $\beta$ -D-glucosaminidase derived from *Diplococcus pneumoniae*, the digested product was eluted at the same position as that of the standard Man<sub>5</sub>GlcNAc<sub>2</sub>-PA in the size-fractionation HPLC (FIG 14B-III). Even when treated with  $\alpha$ -1,2 mannosidase derived from *Aspergillus saitoi*, the elution

position did not shift (FIG 14B-IV). However, when treated with jack bean  $\alpha$ -mannosidase, it was eluted at the same position as that of standard Man<sub>7</sub>GlcNAc<sub>2</sub>-PA in the size-fractionation HPLC (FIG 14B-V). This indicates the removal of four mannose residues in the non-reducing terminus. These results indicate that in the PA sugar chain, none of five mannose residues are  $\alpha$ 1,2 linked to the mannose residue which are  $\alpha$ 1,3 binding. The exoglycosidase digestion, two-dimensional sugar chain mapping, and IS-MS/MS analysis indicate a sugar chain structure of GalGNM5 as shown by L in FIG 15.

FIG 20 summarizes the above results regarding the structure of N-linked glycans and the ratio of each N-linked glycan in GT6 cell line along with those in wild-type BY2 cell line determined similarly. In FIG 20,  $\square$  denotes GlcNAc,  $\bigcirc$  denotes mannose,  $\bullet$  denotes galactose,  $\square$  with hatched lines therein denotes xylose, and  $\bigcirc$  with dots therein denotes fucose respectively.

In GT6 cell line, the isomers Man7-, Man6- and Man5GlcNAc<sub>2</sub> were observed. Because those high-mannose type oligosaccharides will be substrates for  $\beta$  1,4-galactosyltransferase (Gal T), introduction of GlcNAc I, Man I and Man II cDNAs can more efficiently lead the oligosaccharide Man7-5GlcNAc<sub>2</sub> to GlcNAcMan3GlcNAc<sub>2</sub>, which can be a substrate of GalT (FIG 21).

*A. thaliana* *cgli* mutant, that lacks GnT I, can not synthesize complex type N-glycans (von Schaewen, A., Sturm, A., O'Neill, J., and Chrispeels, MJ., Plant Physiol., 1993 Aug;102(4):1109-1118, Isolation of a mutant Arabidopsis plant that lacks N-acetyl glucosaminyl transferase I and



is unable to synthesize Golgi-modified complex N-linked glycans). Complementation with the human GnT I in the *cgli* mutant indicated that the mammalian enzyme could contribute the plant N-glycosylation pathway (Gomez, L. and Chrispeels, M.J., Proc. Natl. Acad. Sci. USA 1994 March 1;91(5):1829-1833, Complementation of an *Arabidopsis thaliana* mutant that lacks complex asparagine-linked glycans with the human cDNA encoding N-acetylglucosaminyltransferase I.) Furthermore, GnT I cDNA isolated from *A. thaliana* complemented N-acetylglucosaminyltransferase I deficiency of CHO Lec1 cells (Bakker, H., Lommen, A., Jordi, W., Stiekema, W., and Bosch, D., Biochem. Biophys. Res. Commun., 1999 Aug 11;261(3):829-32, An *Arabidopsis thaliana* cDNA complements the N-acetylglucosaminyltransferase I deficiency of CHO Lec1 cells). cDNAs encoding human Man I and Man II were isolated and sequenced (Bause, E., Bieberich, E., Rolfs, A., Volker, C. and Schmidt, B., Eur J Biochem 1993 Oct 15;217(2):535-40, Molecular cloning and primary structure of Man9-mannosidase from human kidney; Tremblay, L.O., Campbell, Dyke, N. and Herscovics, A., Glycobiology 1998 Jun;8(6):585-95, Molecular cloning, chromosomal mapping and tissue-specific expression of a novel human alpha 1,2-mannosidase gene involved in N-glycan maturation; and Misago, M., Liao, Y.F., Kudo, S., Eto, S., Mattei, M.G., Moremen, K.W., Fukuda, M.N., Molecular cloning and expression of cDNAs encoding human alpha-mannosidase II and a previously unrecognized alpha-mannosidase IIx isozyme). Human Man I has two isozymes, Man IA and Man IB, and the nucleotide structure of isozymes' cDNA was shown (Bause, E., et al., and Tremblay, L.O., *supra*). By transforming these cDNAs into the BY cell line, an efficient cell line producing human-type glycoprotein, can

be obtained.  $\beta$  1,4-Galactosyltransferase (Gal T) uses UDP-galactose as a donor substrate and GlcNAc2Man3GlcNAc2 as an acceptor substrate. Efficient supply of UDP-galactose will enhance the Gal T enzyme reaction, and more  
5 galactosylated oligosaccharide will be produced (FIG 22).

(Example 7) Structural Analysis of the Sugar Chains on the HRP in the Double Transformant GT6-HRP Cells

A crude cell lysate was obtained from the homogenate of 50  
10 g of cultured GT6-HRP cells or control BY2-HRP cells grown for seven days, respectively. This crude cell lysate solution was applied to a CM Sepharose FF column (1 x 10 cm) (Pharmacia Co., Ltd.) equilibrated with 10 mM of sodium phosphate buffer (pH 6.0). After washing the column, the  
15 eluted peroxidase was measured at an absorbance of 403 nm. The pooled fraction was concentrated using an ultrafilter (molecular weight cut off: 10,000, Advantec Co., Ltd.), dialyzed against 50 mM of a sodium phosphate buffer (pH 7.0), and then applied to an equilibrated benzhydroxaminic  
20 acid-agarose affinity column (1 x 10 cm) (KemEn Tech, Denmark). After the column was washed in 15 volumes of 50 mM of sodium phosphate buffer (pH 7.0), the absorbed HRP was eluted using 0.5 M boric acid prepared in the same buffer. The peroxidase active fraction obtained was then pooled,  
25 dialyzed, and concentrated.

The purified HRP prepared from the double transformant GT6-HRP cells or BY2-HRP cells was applied to a 1 x 10 cm  
30 RCA<sub>120</sub>-agarose column. The column was then washed with 15 volumes of 10 mM ammonium acetate (pH 6.0). The absorbed proteins were then eluted and assayed using conventional methods.

Lectin staining was then performed on the purified HRP eluted from RCA<sub>120</sub> affinity chromatography whose specificity is specific to  $\beta$ 1,4 linkage galactose. The lectin RCA<sub>120</sub> was bound to only the HRP produced by the transformed cell GT6-HRP. Because the lectin binding was dramatically reduced by preincubation with the galactose which can compete with the lectin (FIG 16b-III), the binding is carbohydrate specific. Even when the purified HRP is pre-treated with *D. pneumoniae*  $\beta$ -galactosidase, the RCA<sub>120</sub> binding was inhibited. These results indicate RCA bound specifically to  $\beta$ 1,4-linked galactose at the non-reducing end of N-linked glycan on HRP. The absence of RCA-bound glycoproteins in the BY2-HRP cells indicates that these cells can not transfer the  $\beta$ 1,4 linked galactose residue to the non-reducing terminus of the HRP glycan.

Reverse-phase HPLC of PA derivatives derived from HRP purified using RCA<sub>120</sub> indicated that the sugar chains on the HRP proteins purified from the GT6-HRP appear as a single peak (FIG 17). In the reverse phase HPLC, a Cosmosil 5C18-P column or Asahipak NH2P column was used in a Jasco 880-PU HPLC device with a Jasco 821-FP Intelligent Spectrofluorometer. Neither bound proteins nor detectable peaks were observed in the HRP fractions purified from BY2-HRP. The peak obtained from the GT6-HRP in the size-fractionation chromatography was homogenous. The two-dimensional mapping analysis of the peak and chromatography of the peak at the same time with standard sugar chain indicated that the oligosaccharide contained in the peak was Gal<sub>1</sub>GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>-PA. The confirmation of this structure was provided using continuous exoglycosidase digestion. The standard sugar chains used were a sugar chains prepared previously (Kimura, Y. et al.,

Biosci. Biotech. Biochem. 56 (2), 215-222, 1992) or purchased (Wako Pure Chemical, Industries, Ltd. Osaka and Takara Shuzo Co., Ltd.).

5 The PA sugar chain digested with  $\beta$ -galactosidase (*D. pneumoniae*) matched the elution position of the standard GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>-PA indicating the removal of a galactose residue  $\beta$ 1,4-linked to a non-reducing terminal GlcNAc. Further digestion with *D. pneumoniae* N-acetyl- $\beta$ -D-glucosaminidase of  $\beta$ -galactosidase-digested products  
10 produced a sugar chain equivalent which is eluted at the same elution position of Man<sub>5</sub>GlcNAc<sub>2</sub>-PA, indicating the removal of a GlcNAc residue  $\beta$ 1,2 linked to a non-reducing terminal mannose residue. The removed GlcNAc residue is  
15 believed to be linked to  $\alpha$ 1,3 mannose linked to a  $\beta$ 1,4 mannose residue in view of the N-linked type processing route of the plant. In order to confirm the linkage position of the GlcNAc residue, Man<sub>5</sub>GlcNAc<sub>2</sub>-PA (M5) was incubated with  
20  $\alpha$ 1,2 mannosidase derived from *Aspergillus saitoi*. As expected, an elution position shift was not detected, confirming M5 has the structure Man $\alpha$ 1-6(Man $\alpha$ 1,3)Man $\alpha$ 1-6(Man $\alpha$ 1,3)Man $\beta$ 1,4GlcNAc $\beta$ 1,4GlcNAc as predicted. When the sugar chain was digested using jack bean  $\alpha$ -mannosidase, it was eluted at the same elution positions  
25 of known Man<sub>1</sub>GlcNAc<sub>2</sub>-PA. Therefore, the sugar chain structure corresponded to Man $\alpha$ 1-6(Man $\alpha$ 1,3)Man $\alpha$ 1-6(Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3)Man $\beta$ 1,4GlcNAc $\beta$ 1,4GlcNAc (Gal<sub>1</sub>GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNA<sub>2</sub>). These results indicate that the sugar chain in GT6 cell has the structure shown in FIG 15  
30 and that the sugar chain structure on an HRP protein derived from the double transformant GT6-HRP is Man $\alpha$ 1-6(Man $\alpha$ 1,3)Man $\alpha$ 1-6(Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3)Man $\beta$ 1,4GlcNAc $\beta$ 1,4GlcNAc (Gal<sub>1</sub>GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNA<sub>2</sub>).

Similarly, the galactosylated N-glycan on HRP derived from the transformant GT6-HRP cells did not react with an antiserum which has been shown to specifically react with  $\beta$ 1,2 xylose residue indicative of plant N-glycans. This indicates that one of the sugar residues shown to be antigenic in complex plant glycan, i.e., xylose residue, is not present (Garcia-Casado, G. et al., Glycobiology 6 (4): 471,477, 1996) (FIG 18).

#### INDUSTRIAL APPLICABILITY

The present invention provides a method for manufacturing a glycoprotein with a human-type sugar chain. It also provides plant cells that have a sugar chain adding mechanism able to perform a reaction in which a galactose residue is transferred to a acetylglucosamine residue on the non-reducing terminal, wherein the sugar chain adding mechanism is capable of joining a sugar chain which contains a core sugar chain and an outer sugar chain, wherein the core sugar chain consists essentially of a plurality of mannose and acetylglucosamine, and the outer sugar chain contains a terminal sugar chain portion containing a galactose on the non-reducing terminal. The present invention further provides a glycoprotein with a human-type sugar chain obtained by the present invention. A glycoprotein with a mammalian, e.g., human-type sugar chain of the present invention is not antigenic because the glycosylation is a human-type. Therefore, it can be useful for administering to animals including humans.

## CLAIMS

1. A method of manufacturing a glycoprotein having a human-type sugar chain, comprising a step in which a transformed plant cell is obtained by introducing to a plant cell the gene of glycosyltransferase and the gene of an exogenous glycoprotein, and a step in which the obtained transformed plant cell is cultivated.
2. A method according to claim 1, wherein the glycosyltransferase is an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue.
3. A method according to Claim 1, wherein the glycoprotein with a human-type sugar chain comprises a core sugar chain and an outer sugar chain, wherein the core sugar chain comprises a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.
4. A method according to Claim 3, wherein the outer sugar chain has a straight chain configuration.
5. A method according to Claim 3, wherein the outer sugar chain has a branched configuration.
6. A method according to Claim 5, wherein the branched sugar chain portion has a mono-, bi-, tri- or tetra configuration.
7. A method according to Claim 1 through Claim 6, wherein the glycoprotein contains neither fucose nor xylose.

8. A plant cell having a sugar chain adding mechanism which can conduct a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue, wherein the sugar chain adding mechanism adds a sugar chain  
5 containing a core sugar chain and an outer sugar chain, wherein the core sugar chain comprises a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.

10

9. A plant cell according to claim 8, wherein the plant cell is transformed with the gene of a first enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and the  
15 gene of a second enzyme which can enhance the first enzyme.

10. A plant cell according to claim 9, wherein the second enzyme is selected from the group consisting of Mannosidase I, Mannosidase II,  $\beta$ 1,4-Galactosyltransferase (GalT) and  
20 N-acetylglucosaminyltransferase I (GlcNAcI).

11. A plant regenerated from the plant cell of claim 8.

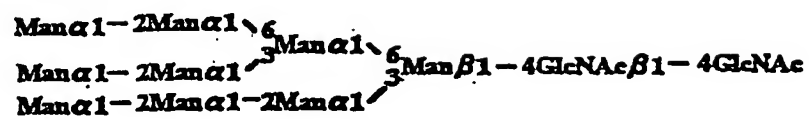
12. A recombinant plant, or portion thereof, that produces  
25 mammalian-like glycoproteins comprising neither fucose or xylose.

13. A glycoprotein with a human-type sugar chain obtained using the method according to Claim 1 through Claim 7.

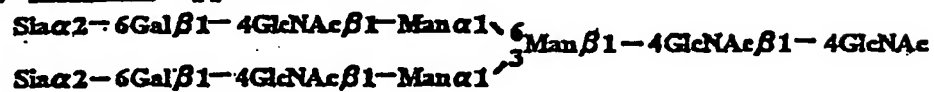
30

14. A plant-produced glycoprotein comprising neither fucose or xylose.

## 1) High Mannose-Type



## 2) Complex-Type



## 3) Hybrid-Type

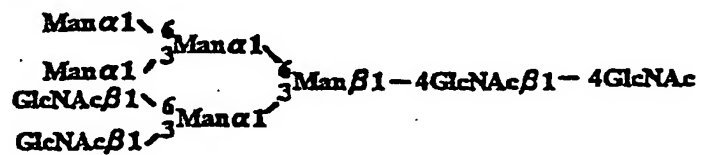


FIG 1



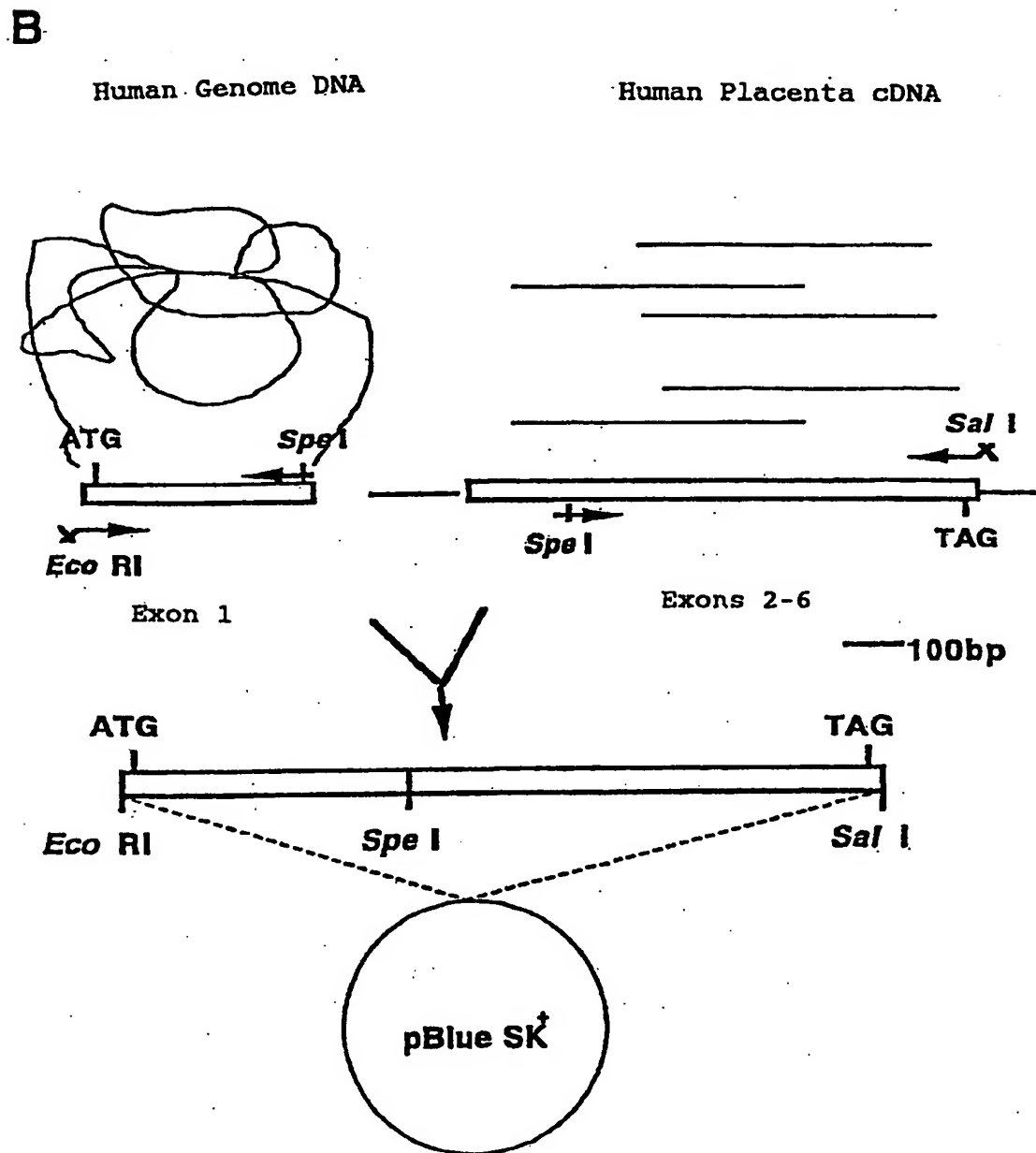
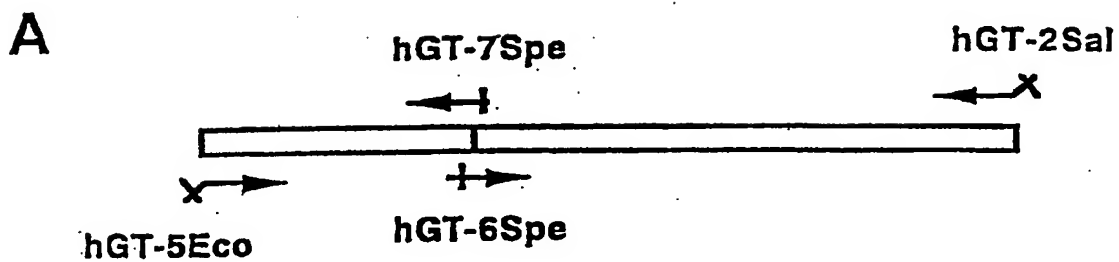


FIG 2

3/21

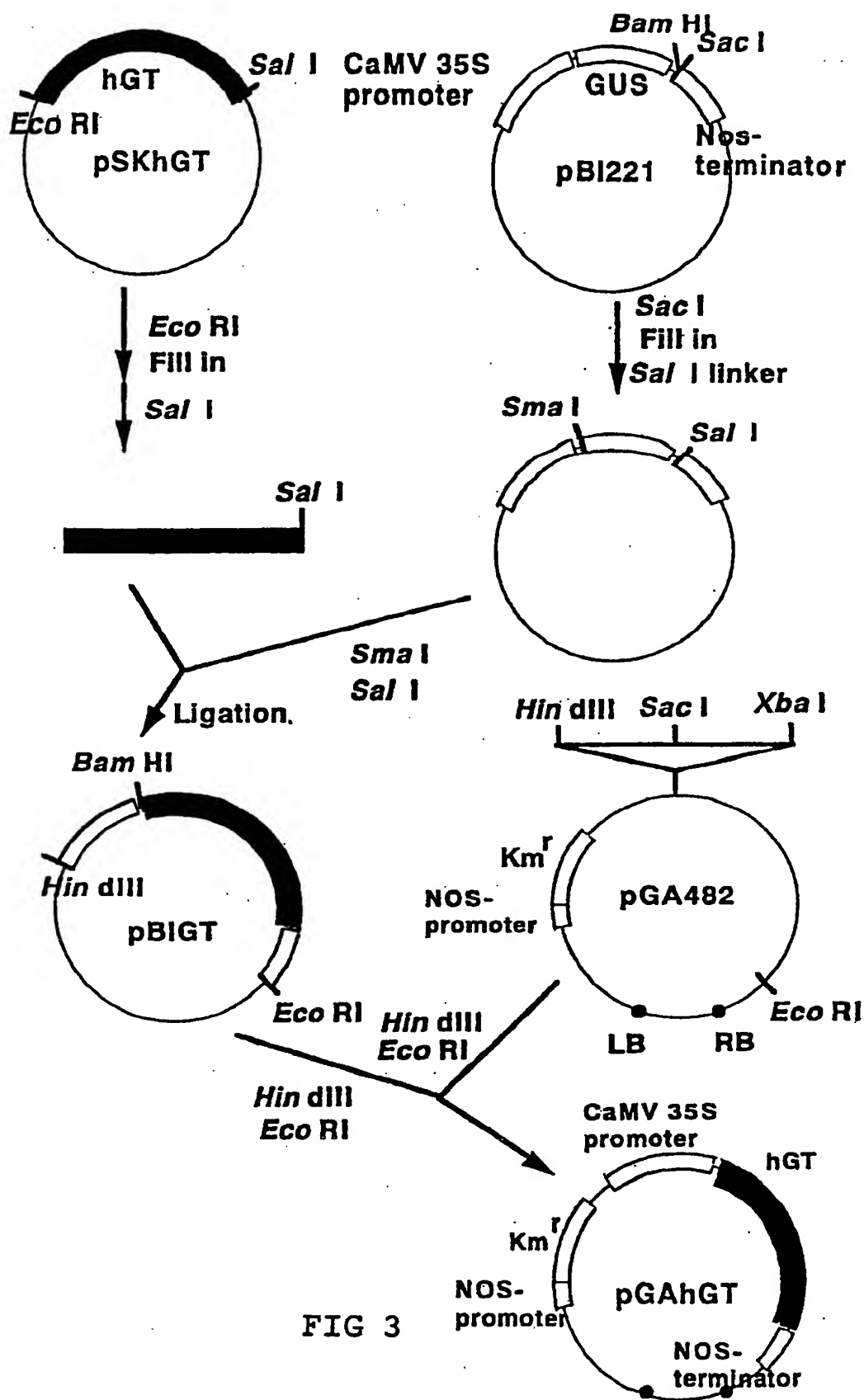


FIG 3

4/21

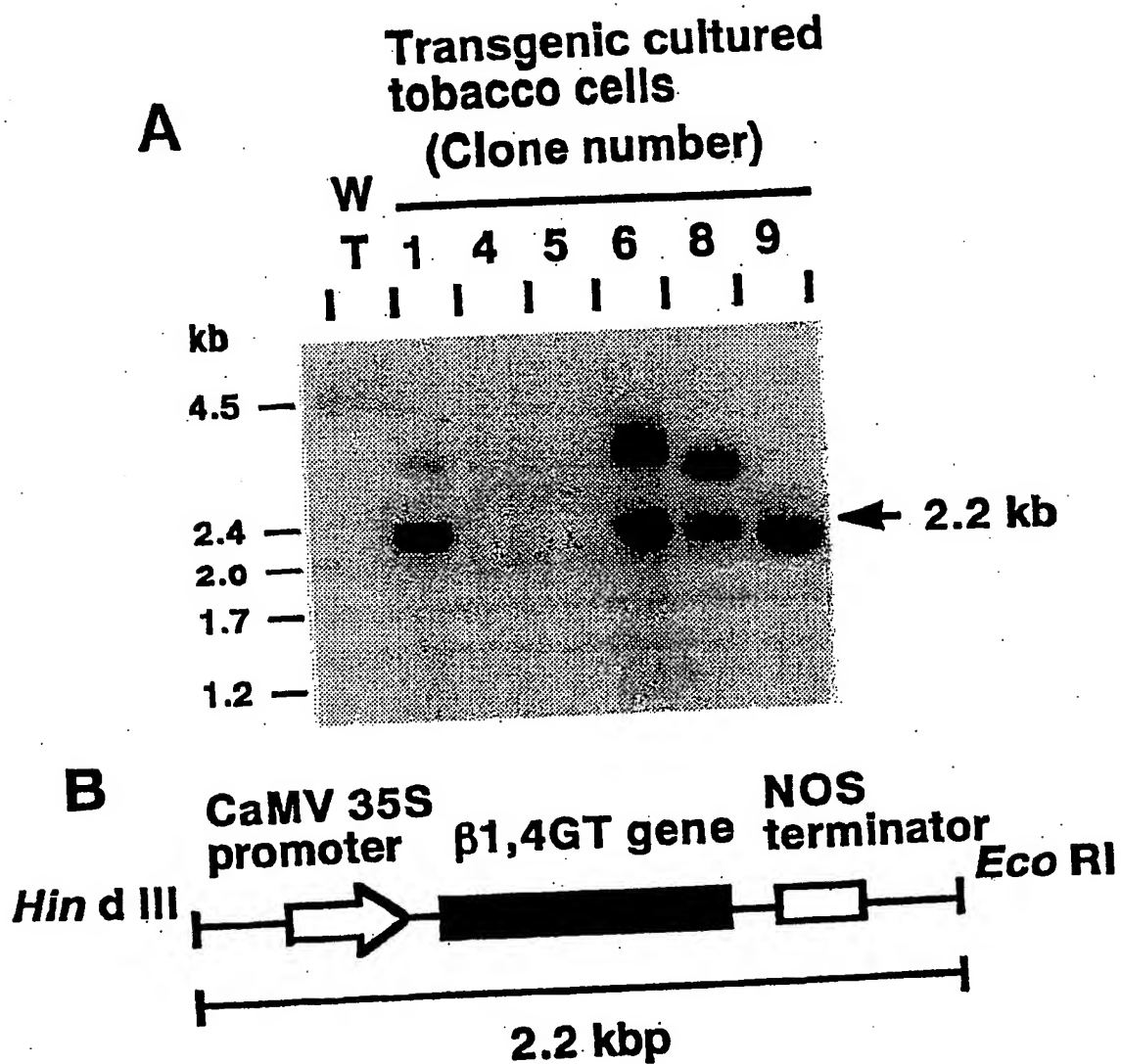
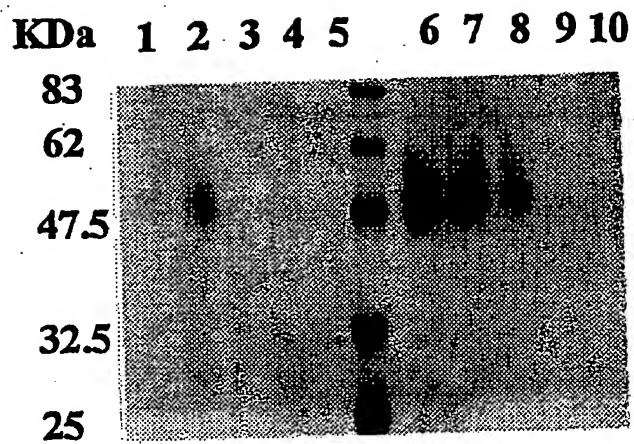


FIG 4



**FIG 5**

6/21

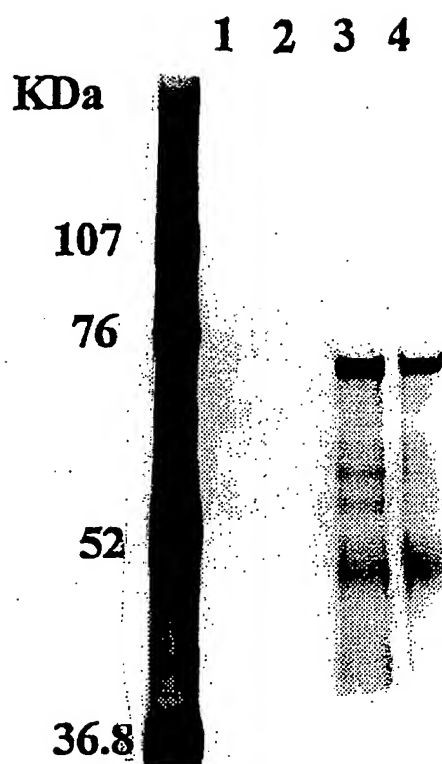


FIG 6

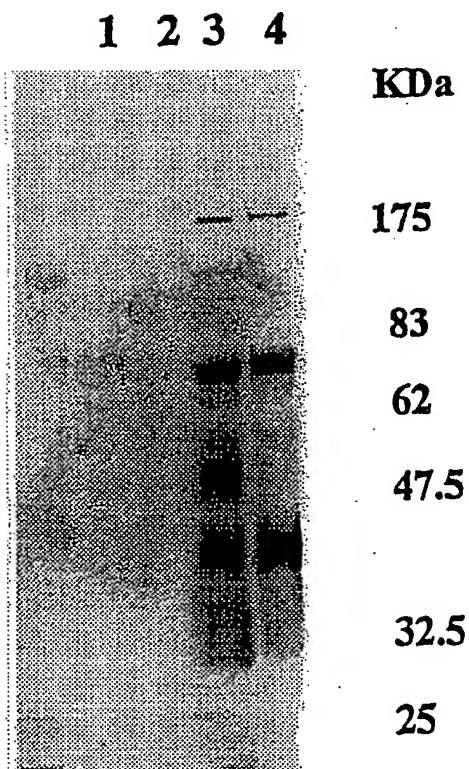


FIG 7

7/21

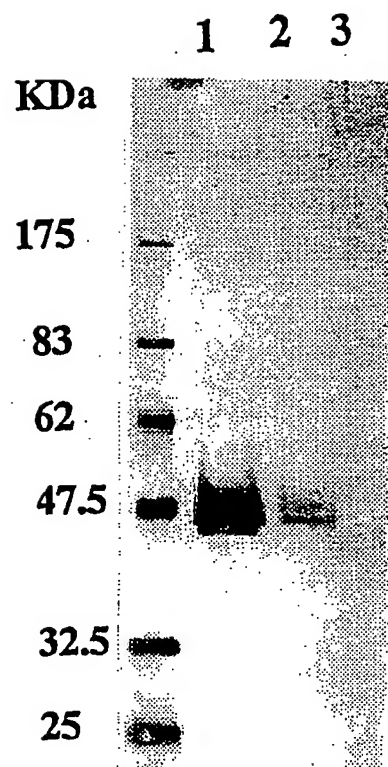


FIG 8

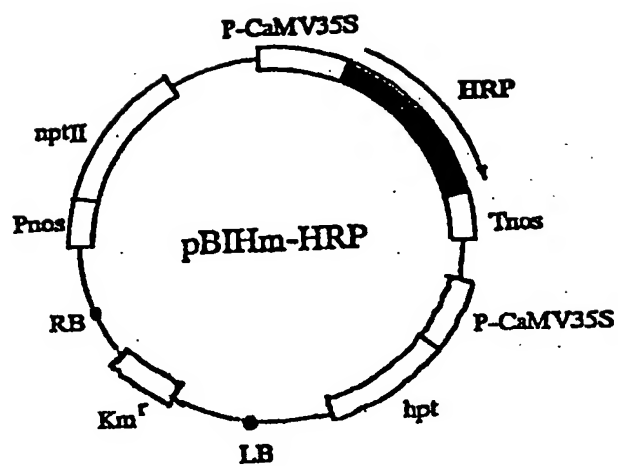


FIG 9

9/21

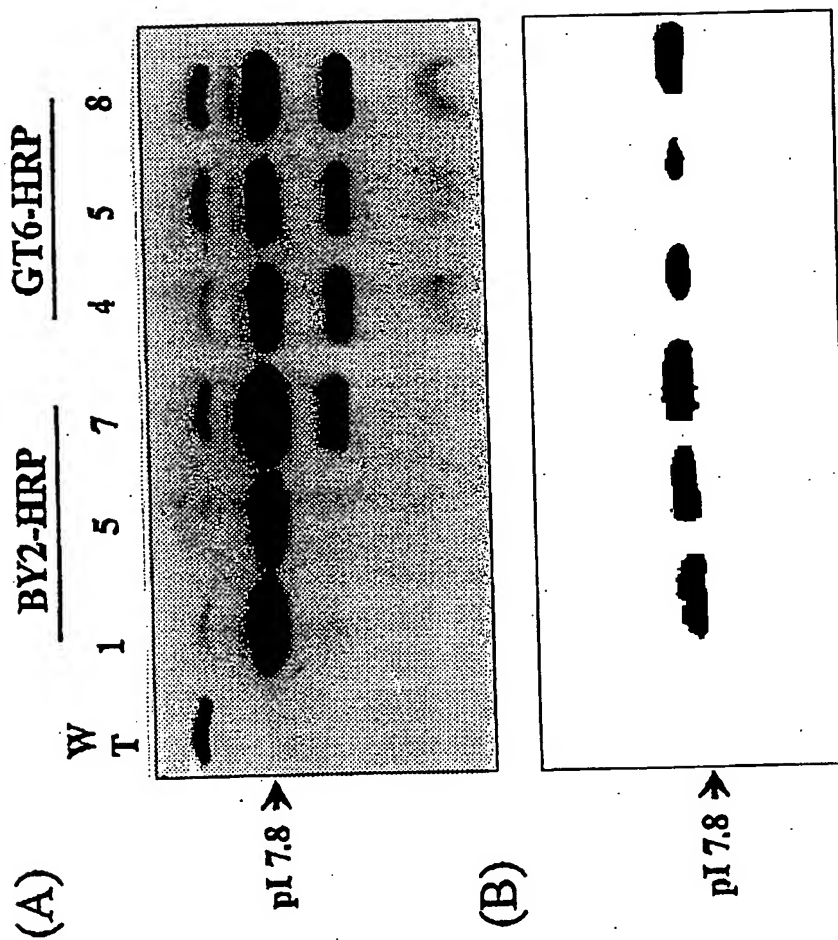


FIG 10



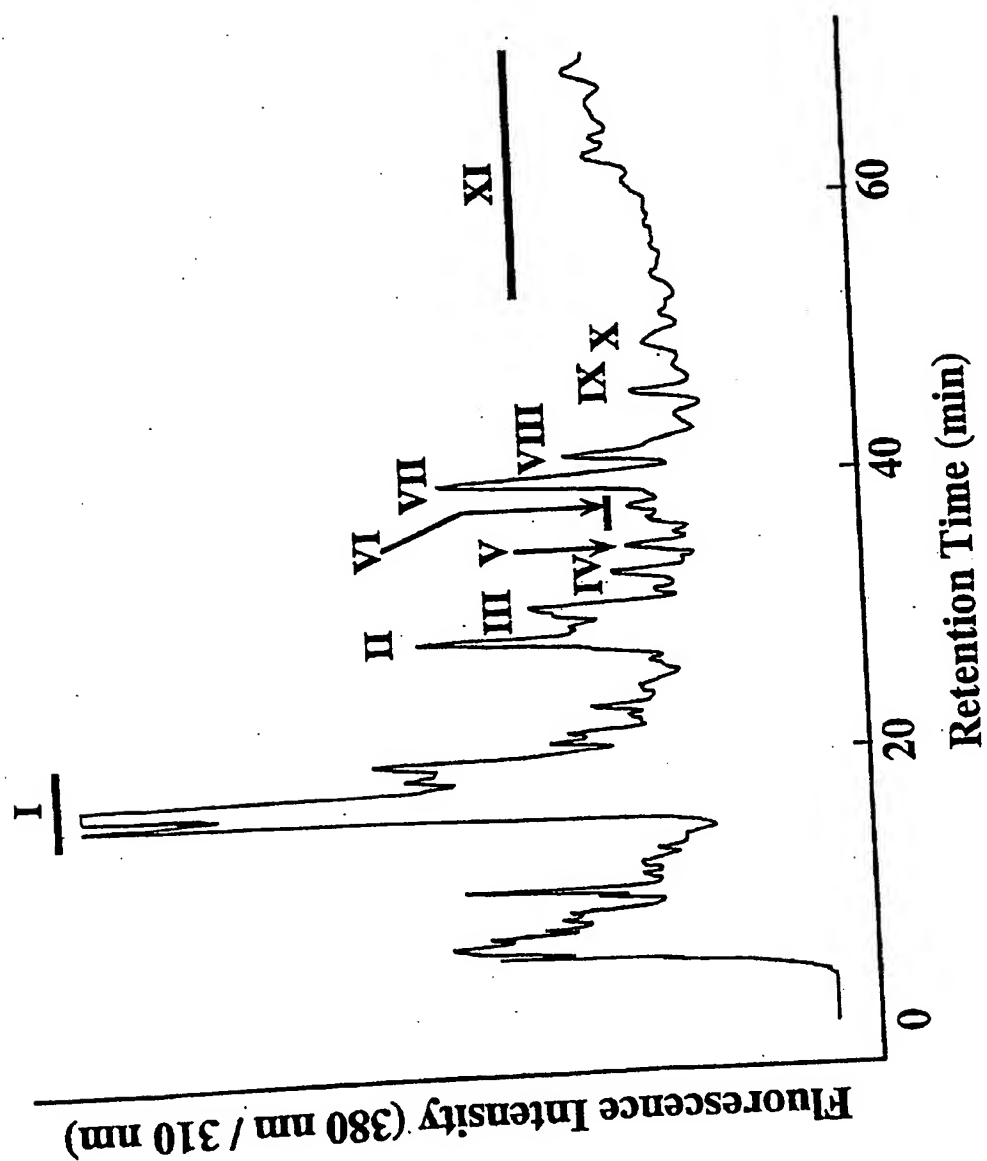


FIG 11

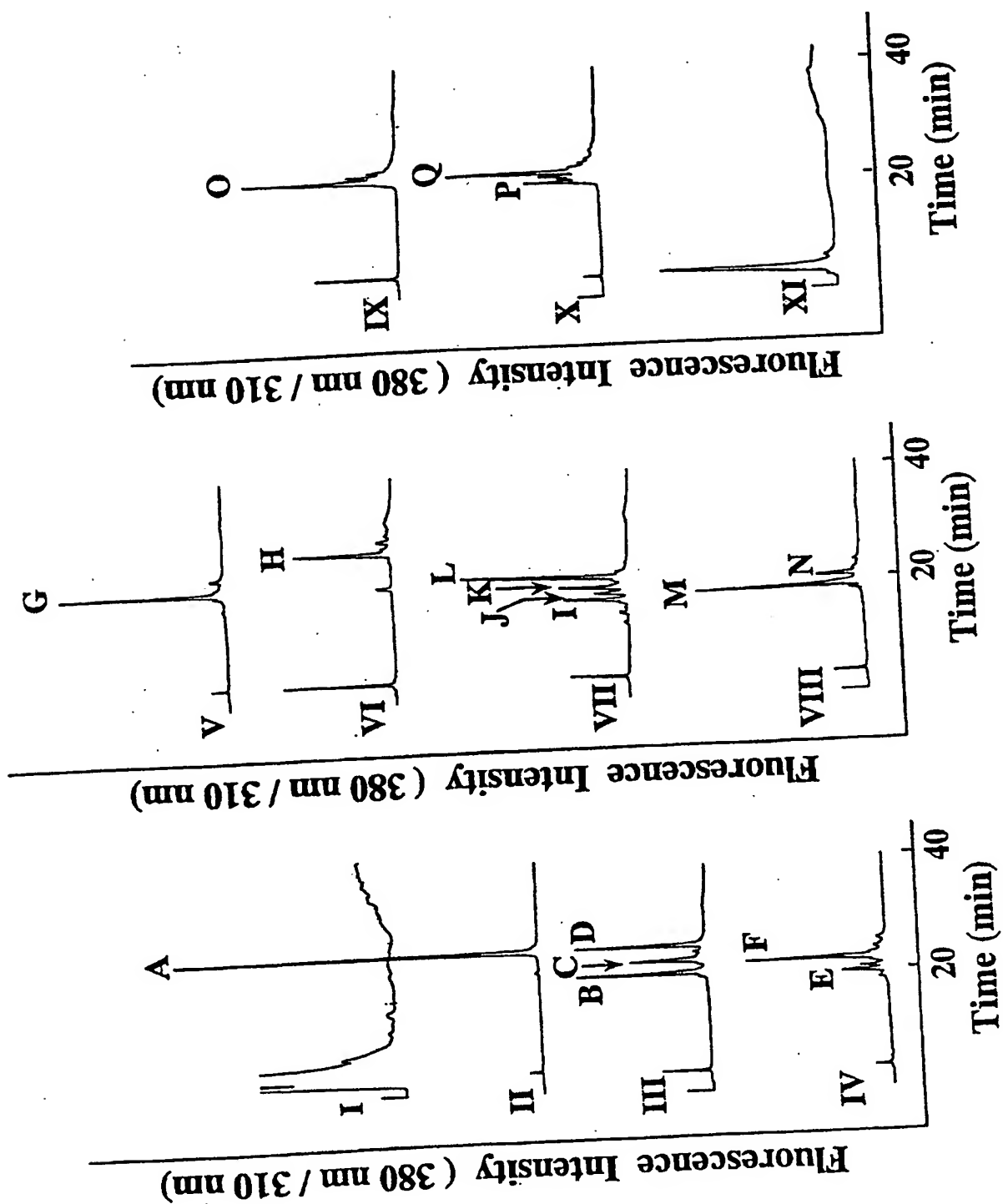


FIG 12

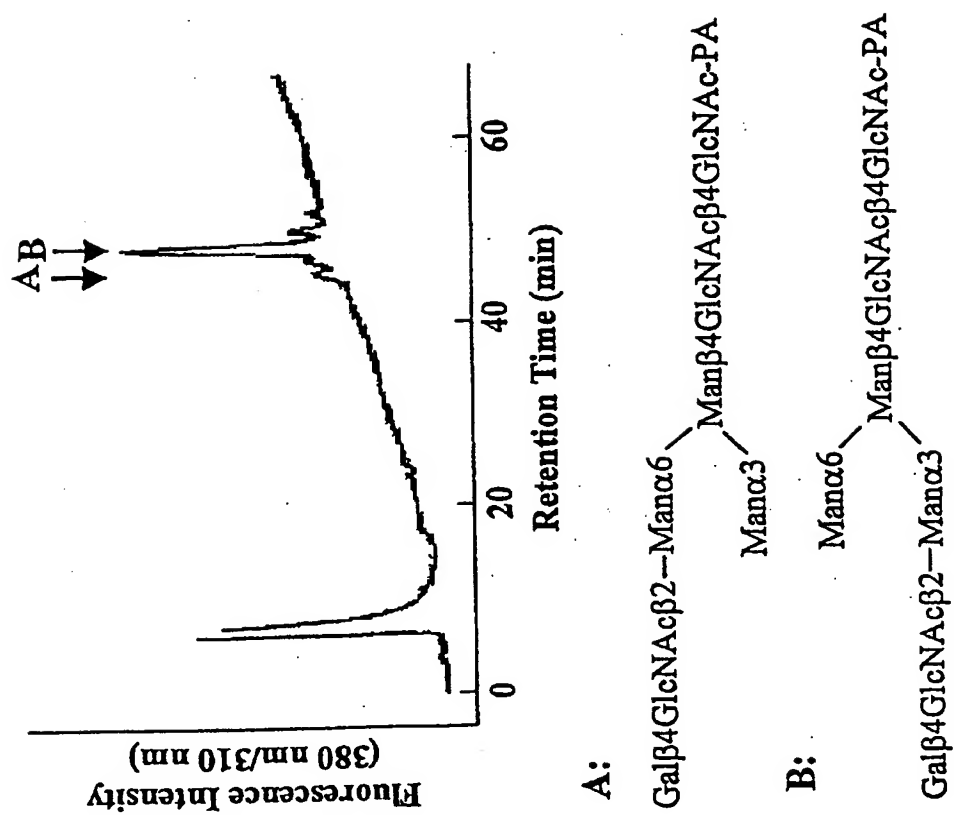


FIG 13

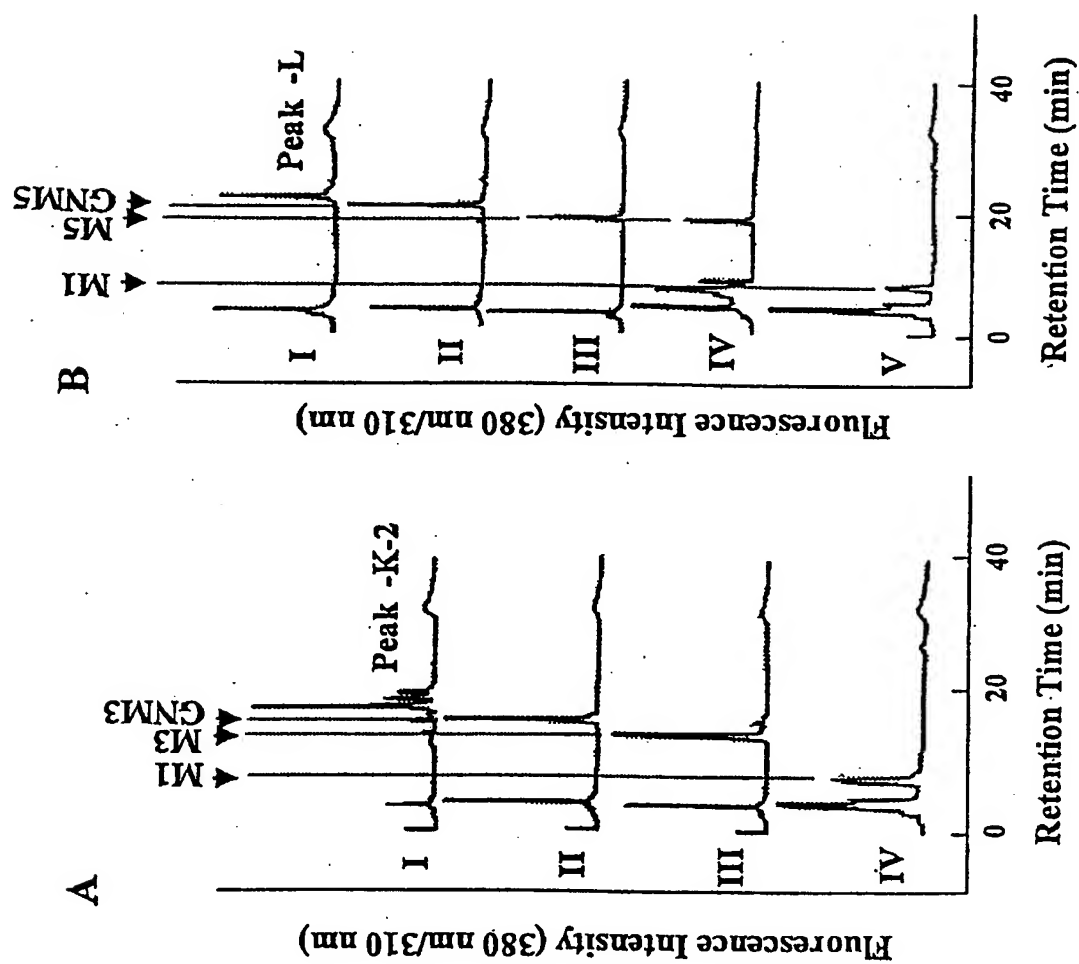


FIG 14

14/21

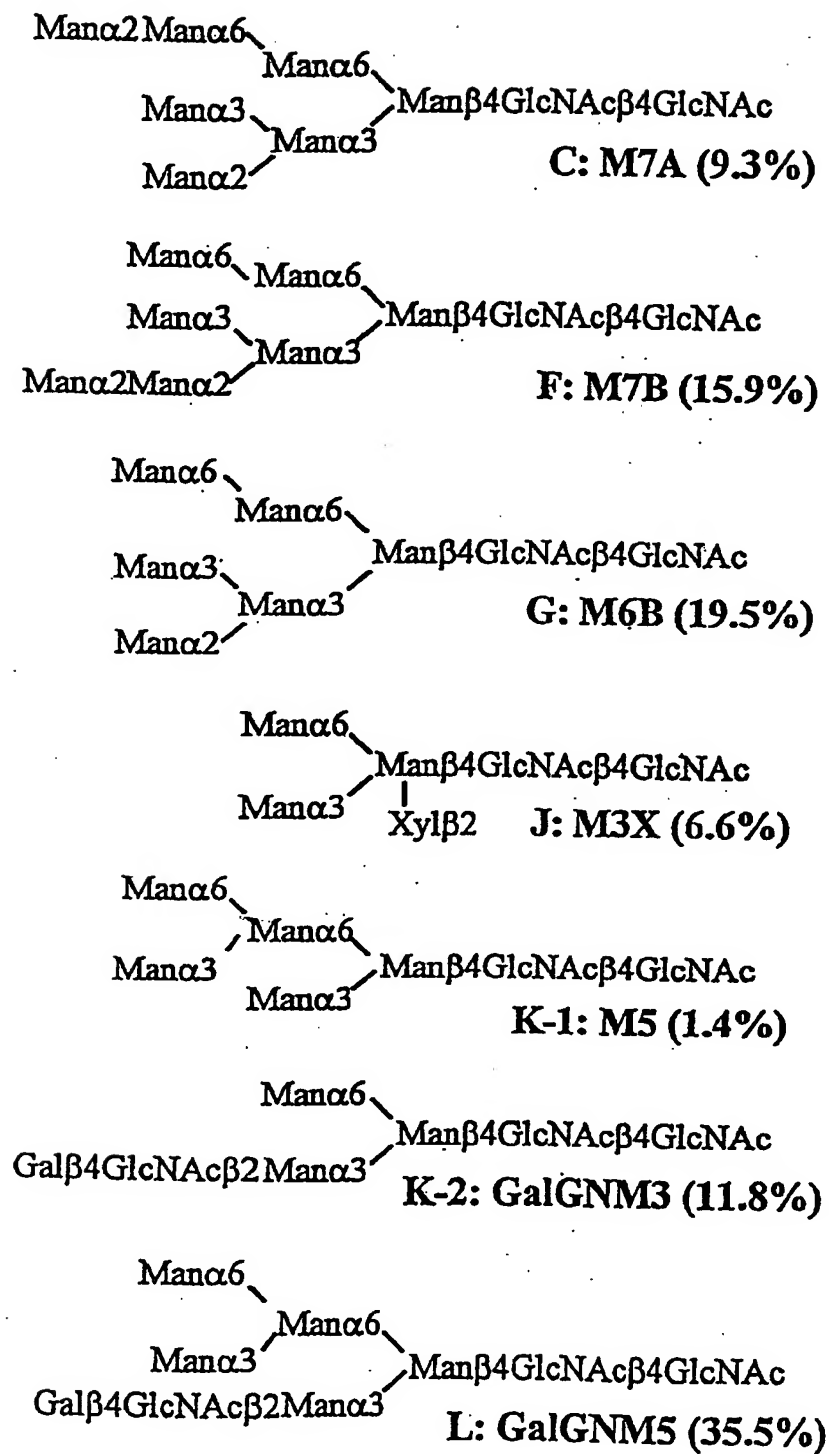


FIG 15

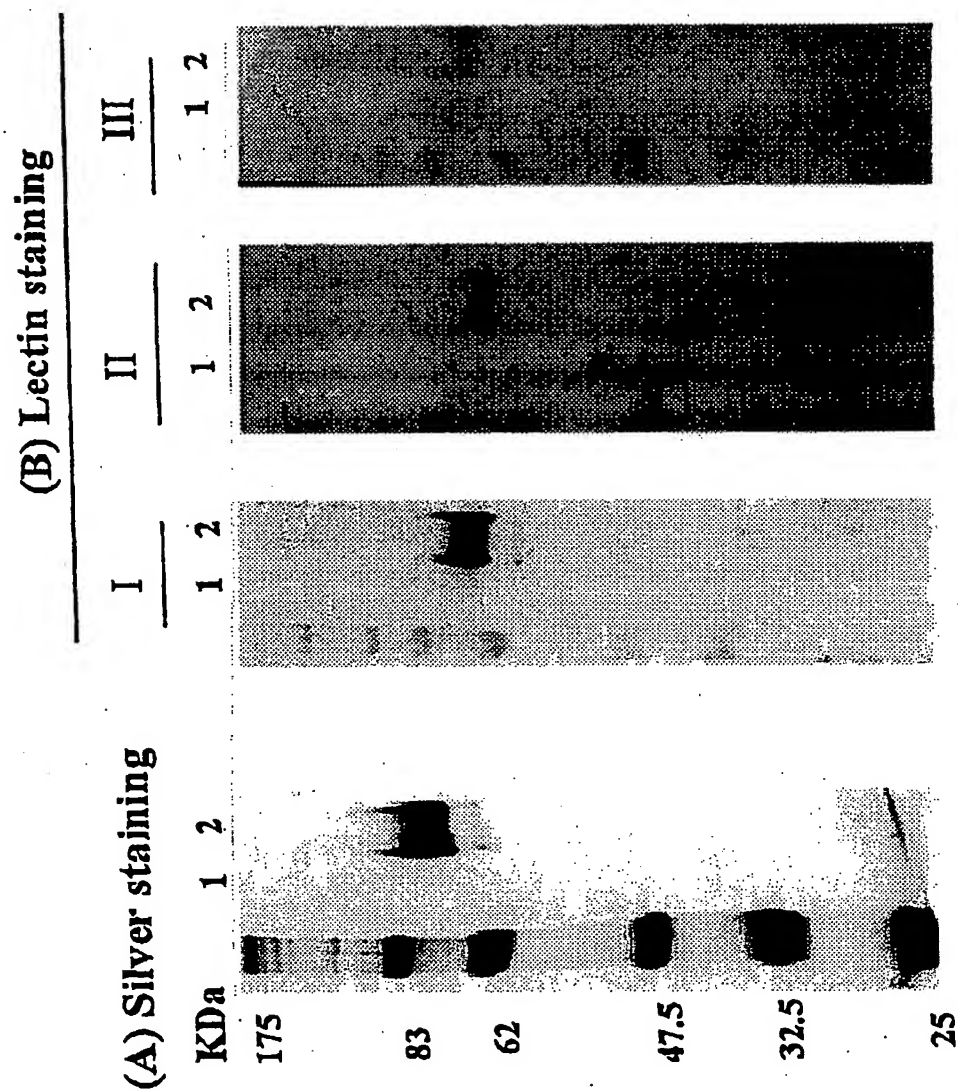


FIG 16

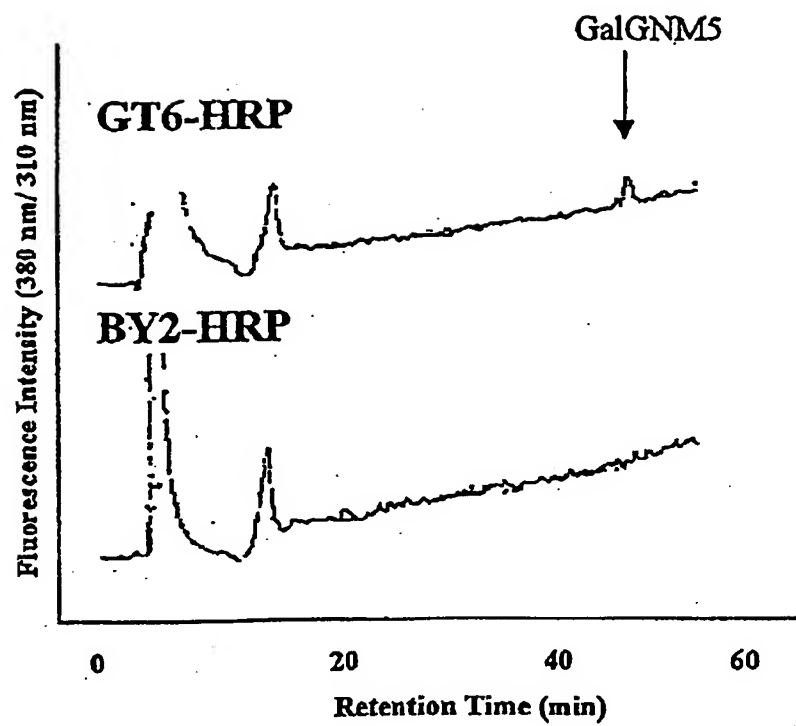


FIG 17

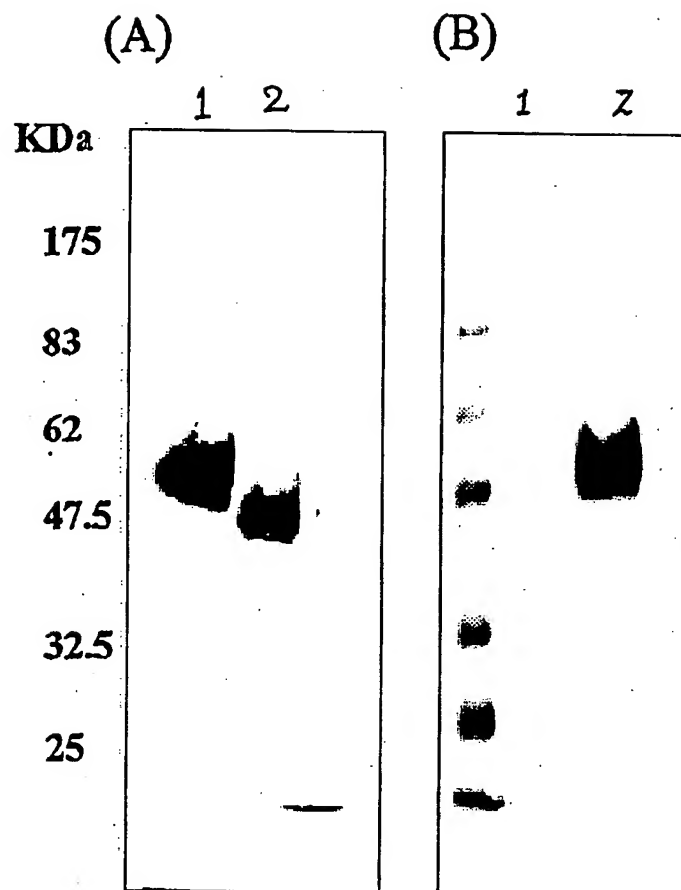


FIG 18



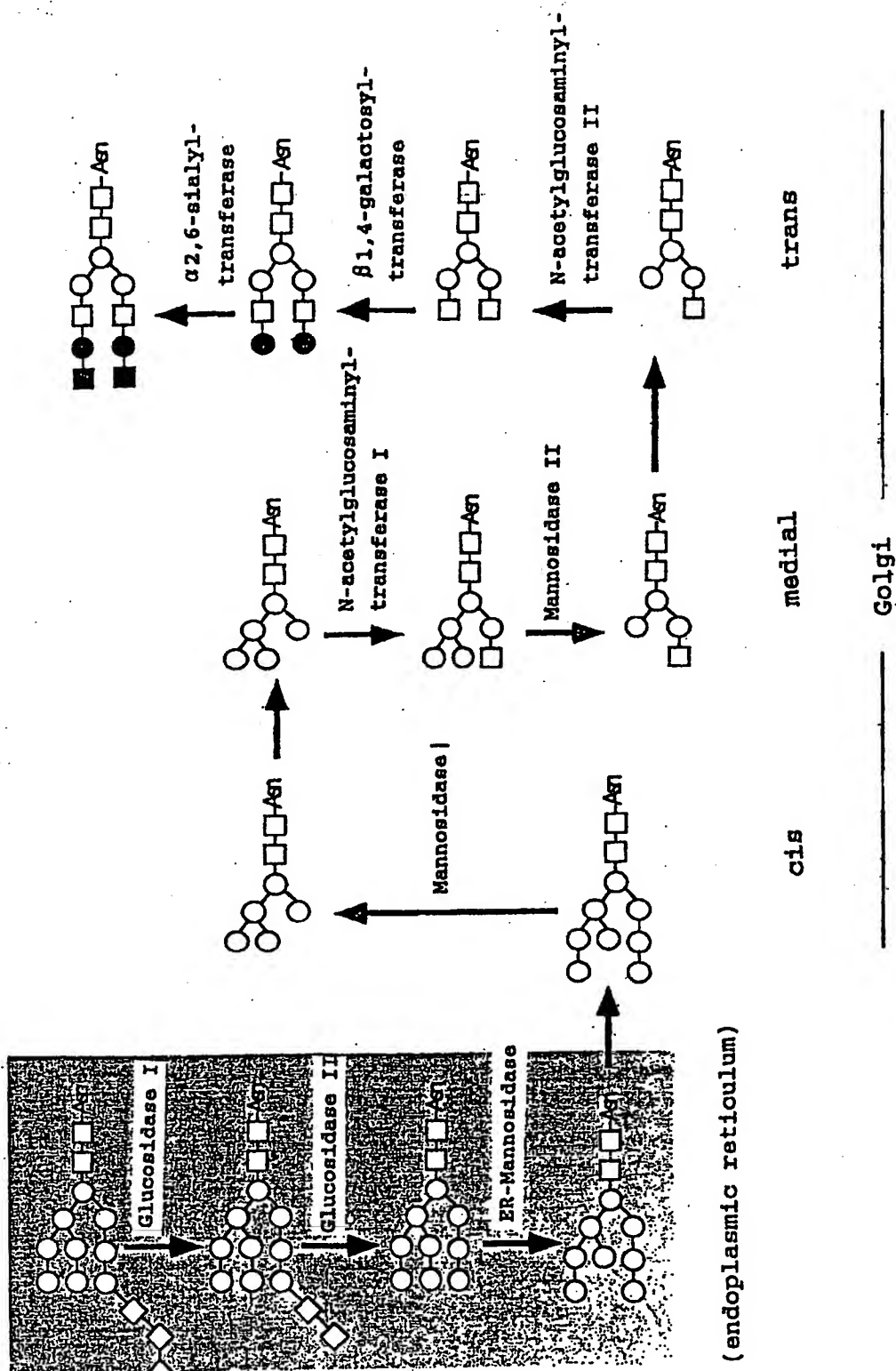


FIG 19

19/21

Wild type; BY2 cell

Transgenic; GT6 cell

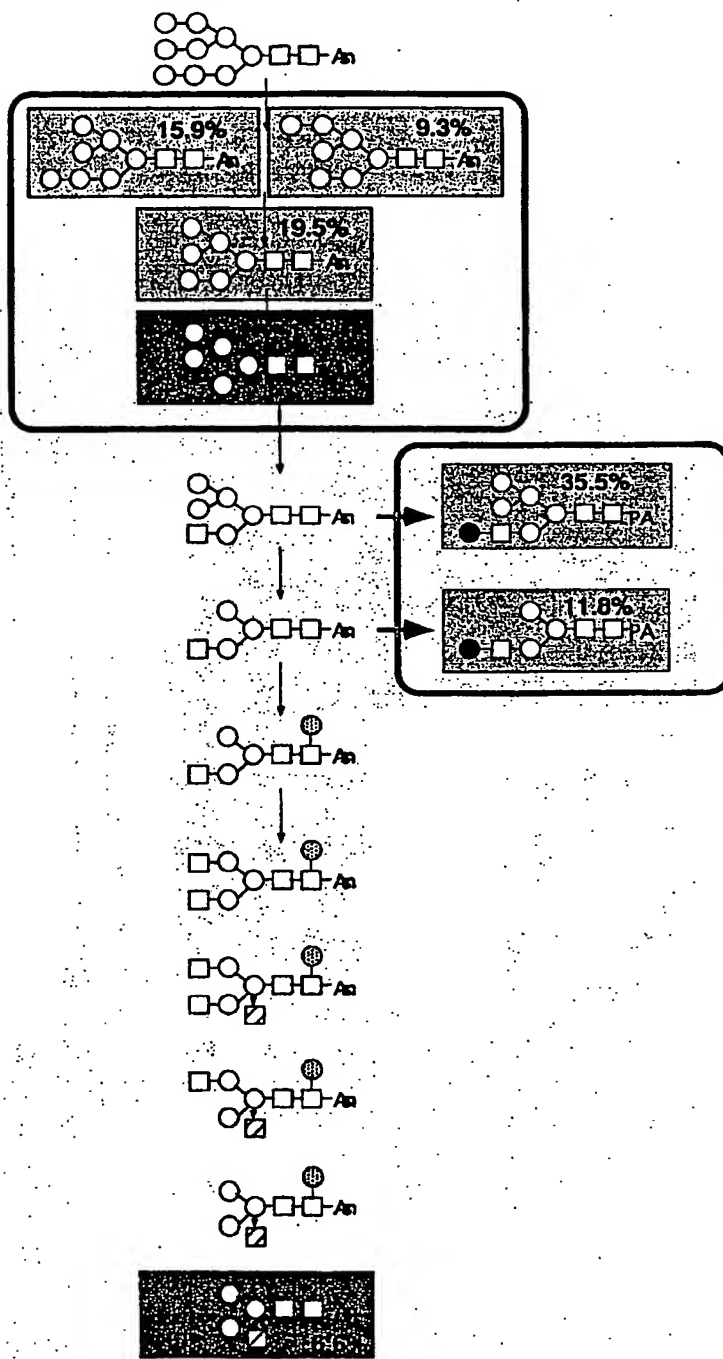
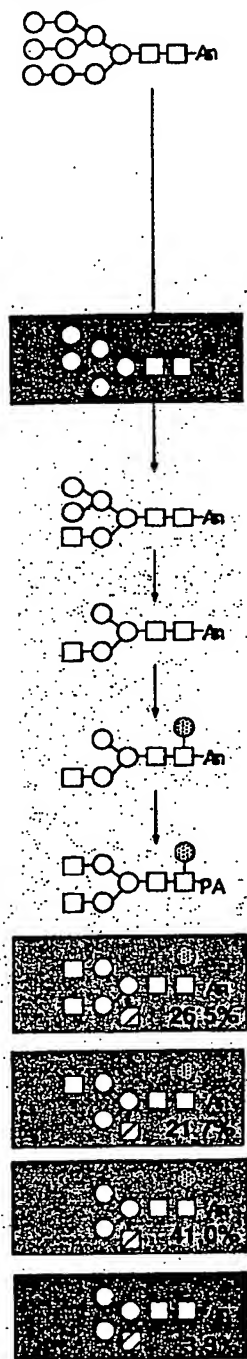
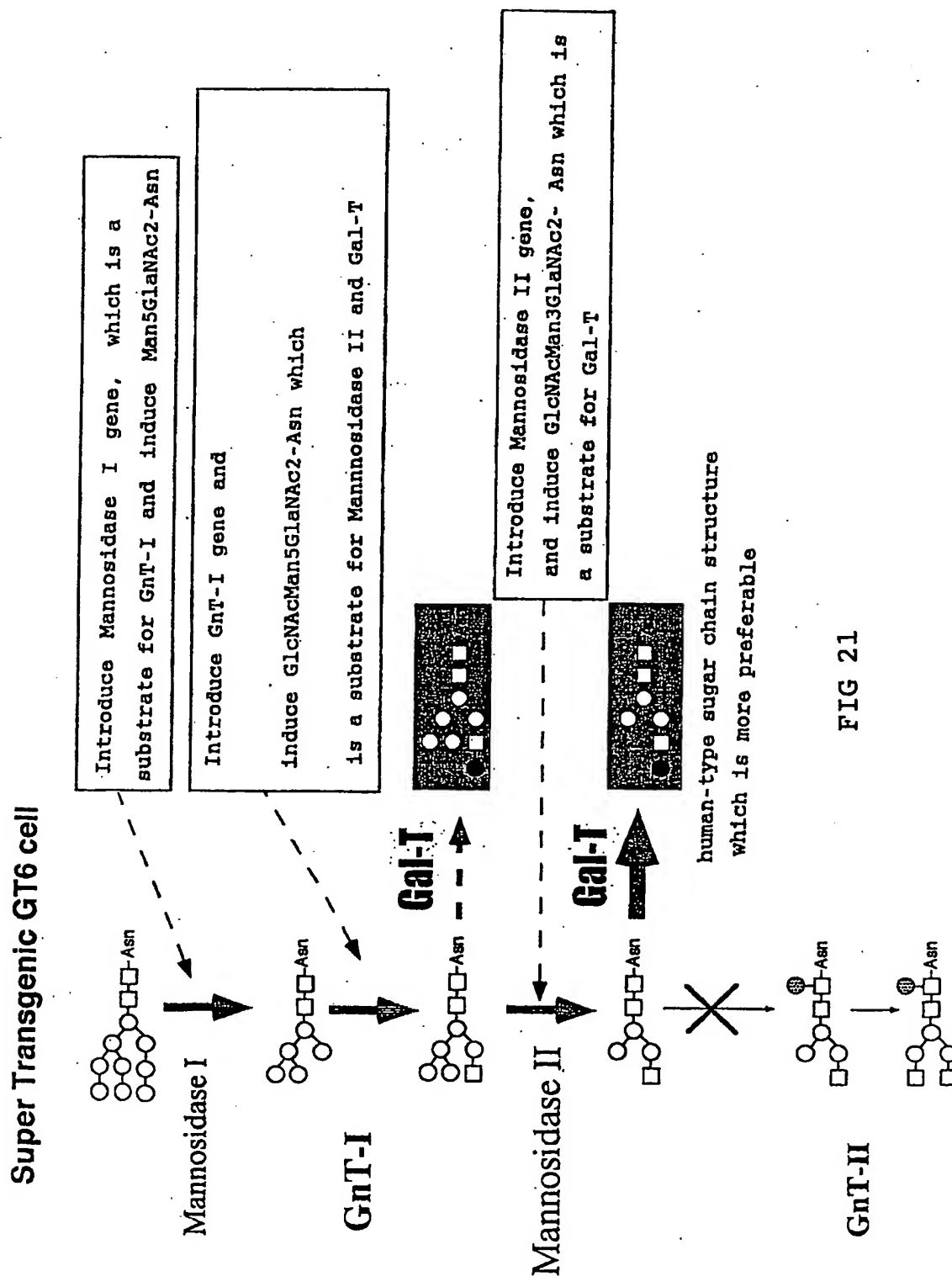


FIG 20

20/21



21/21

UDP-galactose      synthetic      enzyme      group

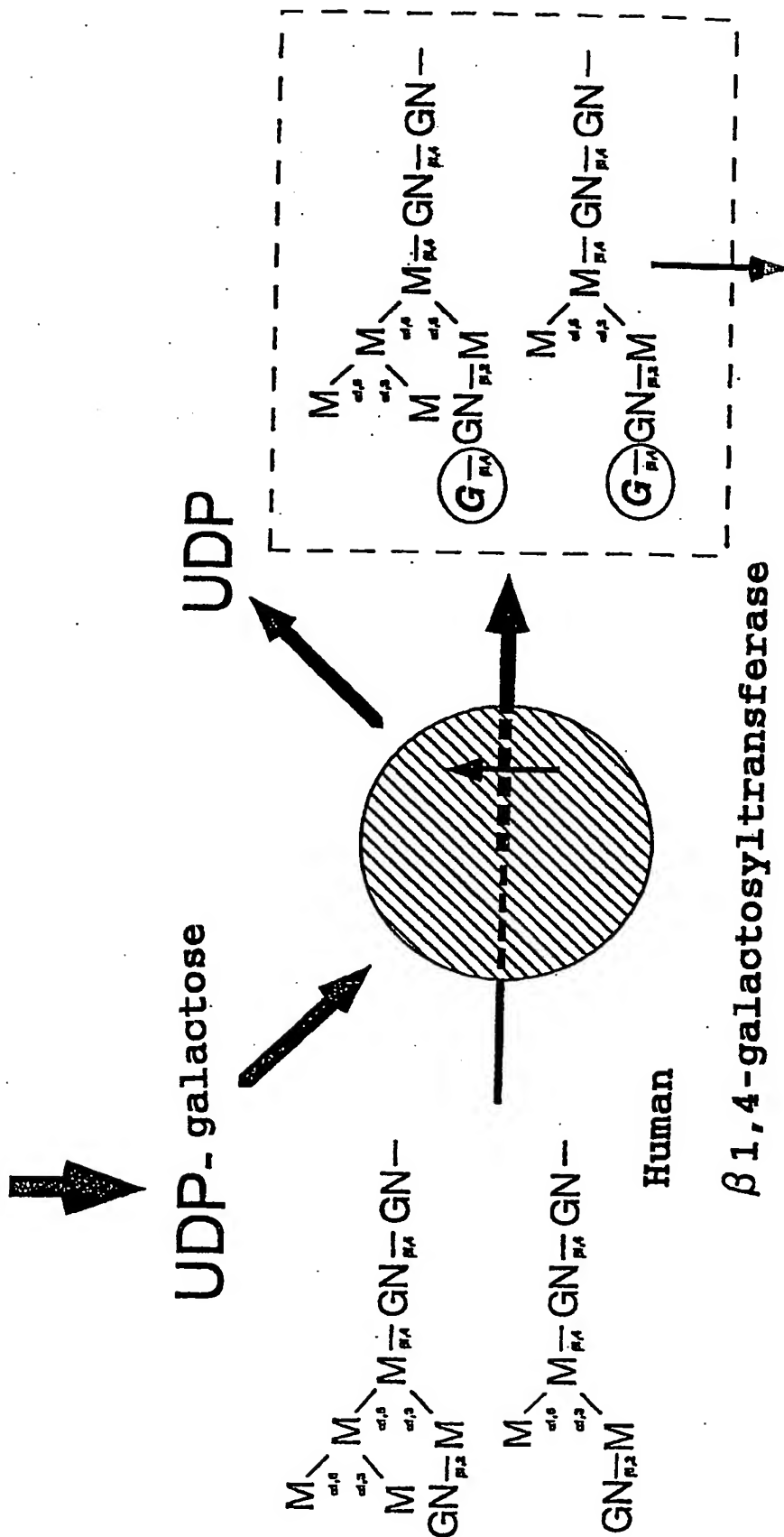


FIG 22

## 1/8

## Sequence Listing

<110> Tatsuji, Seki and Kazuhito, Fujiyama  
<120> Methods for production of glycoproteins  
5       having human-type sugar chains  
<130> J198080401  
<150> JP P1998-350584  
<151> 1998-12-09  
<160> 6  
10   <170> PatentIn Ver. 2.0  
  
      <210> 1  
      <211> 31  
      <212> DNA  
15   <213> Artificial Sequence  
      <220>  
      <223> Description of Artificial Sequence: primer hGT-5Eco  
      <400> 1  
          aaagaattcg cgaigccagg cgcgcgltccc t                   31  
20  
      <210> 2  
      <211> 28  
      <212> DNA  
      <213> Artificial Sequence  
25   <220>  
      <223> Description of Artificial Sequence: primer hGT-2Sal  
      <400> 2  
          tcgategcaa aacctatgic agcigatg                   28  
30  
      <210> 3  
      <211> 29  
      <212> DNA  
      <213> Artificial Sequence

2/8

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer hGT-7Spe

&lt;400&gt; 3

acgggactcc tcaggggcga tgatcataa

29

5

&lt;210&gt; 4

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

10

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer hGT-6Spe

&lt;400&gt; 4

aagactagtg ggccccaigc tgatiga

27

15

&lt;210&gt; 5

&lt;211&gt; 1158

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

20

&lt;221&gt; CDS

&lt;222&gt; (1).. (1155)

&lt;400&gt; 5

atg cca ggc gcg tcc cta cag cgg gcc tgc cgc ctg ctc gtc gcc gtc 48

Met Pro Gly Ala Ser Leu Gln Arg Ala Cys Arg Leu Leu Val Ala Val

25

1

5

10

15

tgc gct ctg cac ctt ggc gtc acc ctc gtt tac tac ctg gct ggc cgc 96

Cys Ala Leu His Leu Gly Val Thr Leu Val Tyr Tyr Leu Ala Gly Arg

20

25

30

30

gac ctg agc cgc ctg ccc caa ctg gtc gga gtc tcc aca ccg ctg cag 144

Asp Leu Ser Arg Leu Pro Gln Leu Val Gly Val Ser Thr Pro Leu Gln

35

40

45

3/8

	ggc ggc tgc aac agt gcc gcc gcc atc ggg cag tcc tcc ggg gag ctc	192
	Gly Gly Ser Asn Ser Ala Ala Ala Ile Gly Gln Ser Ser Gly Gln Leu	
	50 55 60	
5		
	cgg acc gga ggg gcc cgg ccg ccg cct cct cta ggc gcc tcc tcc cag	240
	Arg Thr Gly Gly Ala Arg Pro Pro Pro Pro Leu Gly Ala Ser Ser Gln	
	65 70 75 80	
10		
	ccg cgc ccg ggt ggc gac tcc agc cca gtc gtg gat tct ggc cct ggc	288
	Pro Arg Pro Gly Gly Asp Ser Ser Pro Val Val Asp Ser Gly Pro Gly	
	85 90 95	
15		
	ccc gct agc aac ttg acc tgc gtc cca gtg ccc cac acc acc gca ctg	336
	Pro Ala Ser Asn Leu Thr Ser Val Pro Val Pro His Thr Thr Ala Leu	
	100 105 110	
20		
	tgc ctg ccc gcc tgc cct gag gag tcc ccg cta cta gtg ggc ccc atg	384
	Ser Leu Pro Ala Cys Pro Gln Glu Ser Pro Leu Leu Val Gly Pro Met	
	115 120 125	
25		
	ctg att gag ttt aac atg cct gtg gac ctg gag ctc gtg gca aag cag	432
	Leu Ile Glu Phe Asn Met Pro Val Asp Leu Glu Leu Val Ala Lys Gln	
	130 135 140	
30		
	aac cca aat gtg aag atg ggc ggc cgc tat gcc ccc agg gac tgc gtc	480
	Asn Pro Asn Val Lys Met Gly Gly Arg Tyr Ala Pro Arg Asp Cys Val	
	145 150 155 160	
30		
	tct cct cac aag gtg gcc atc atc att cca ttc cgc aac cgg cag gag	528
	Ser Pro His Lys Val Ala Ile Ile Ile Pro Phe Arg Asn Arg Gln Glu	
	165 170 175	

4/8

	cac ctc aag tac tgg cta tat tat ttg cac cca gtc ctg cag cgc cag	576
	His Leu Lys Tyr Trp Leu Tyr Tyr Leu His Pro Val Leu Gln Arg Gln	
	180 185 190	
5	cag ctg gac tat ggc atc tat gtt atc aac cag gcg gga gac act ata	624
	Gln Leu Asp Tyr Gly Ile Tyr Val Ile Asn Gln Ala Gly Asp Thr Ile	
	195 200 205	
10	ttc aat cgt gct aag ctc ctc aat gtt ggc ttt caa gaa gcc ttg aag	672
	Phe Asn Arg Ala Lys Leu Leu Asn Val Gly Phe Gln Glu Ala Leu Lys	
	210 215 220	
15	gac tat gac tac acc tgc ttt gtg ttt agt gac gtg gac ctc att cca	720
	Asp Tyr Asp Tyr Thr Cys Phe Val Phe Ser Asp Val Asp Leu Ile Pro	
	225 230 235 240	
20	atg aat gac cat aat gcg tac agg tgt ttt tca cag cca cgg cac att	768
	Met Asn Asp His Asn Ala Tyr Arg Cys Phe Ser Gln Pro Arg His Ile	
	245 250 255	
25	ttc gtt gca atg gat aag ttt gga ttc agc cta cct tat gtt cag tat	816
	Ser Val Ala Met Asp Lys Phe Gly Phe Ser Leu Pro Tyr Val Gln Tyr	
	260 265 270	
30	ttt gga ggt gtc tct gct cta agt aaa caa cag ttt cta acc atc aat	864
	Phe Gly Gly Val Ser Ala Leu Ser Lys Gln Gln Phe Leu Thr Ile Asn	
	275 280 285	
30	gga ttt cct aat aat tat tgg ggc tgg gga gga gaa gat gat gac att	912
	Gly Phe Pro Asn Asn Tyr Trp Gly Trp Gly Gly Glu Asp Asp Asp Ile	
	290 295 300	
	ttt aac aga tta gtt ttt aga ggc atg tct ata tct cgc cca aat gct	960



5/8

Phe Asn Arg Leu Val Phe Arg Gly Met Ser Ile Ser Arg Pro Asn Ala  
 305 310 315 320

5     gtg gtc ggg agg tgt cgc atg atc cgc cac tca aga gac aag aaa aat 1008  
       Val Val Gly Arg Cys Arg Met Ile Arg His Ser Arg Asp Lys Lys Asn  
                   325 330 335

10     gaa ccc aat cct cag agg ttt gac cga att gca cac aca aag gag aca 1056  
       Glu Pro Asn Pro Gln Arg Phe Asp Arg Ile Ala His Thr Lys Glu Thr  
                   340 345 350

15     atg ctc tct gal ggt ttg aac tca ctc acc tac cag gtg ctg gat gla 1104  
       Met Leu Ser Asp Gly Leu Asn Ser Leu Thr Tyr Gln Val Leu Asp Val  
                   355 360 365

20     cag aga tac cca ttg tal acc caa atc aca gtg gac atc ggg aca ccg 1152  
       Gln Arg Tyr Pro Leu Tyr Thr Gln Ile Thr Val Asp Ile Gly Thr Pro  
                   370 375 380

25     agc tag 1158  
       Ser  
       385

30     <210> 6  
       <211> 385  
       <212> PRT  
       <213> Homo sapiens  
       <400> 6  
       Met Pro Gly Ala Ser Leu Gln Arg Ala Cys Arg Leu Leu Val Ala Val  
           1 5 10 15  
       Cys Ala Leu His Leu Gly Val Thr Leu Val Tyr Tyr Leu Ala Gly Arg  
           20 25 30

6/8

	Asp	Leu	Ser	Arg	Leu	Pro	Gln	Leu	Val	Gly	Val	Ser	Thr	Pro	Leu	Gln
		35					40					45				
5	Gly	Gly	Ser	Asn	Ser	Ala	Ala	Ala	Ile	Gly	Gln	Ser	Ser	Gly	Glu	Leu
		50				55					60					
	Arg	Thr	Gly	Gly	Ala	Arg	Pro	Pro	Pro	Pro	Leu	Gly	Ala	Ser	Ser	Gln
	65				70					75					80	
10	Pro	Arg	Pro	Gly	Gly	Asp	Ser	Ser	Pro	Val	Val	Asp	Ser	Gly	Pro	Gly
					85					90				95		
	Pro	Ala	Ser	Asn	Leu	Thr	Ser	Val	Pro	Val	Pro	His	Thr	Thr	Ala	Leu
15					100				105					110		
	Ser	Leu	Pro	Ala	Cys	Pro	Glu	Glu	Ser	Pro	Leu	Leu	Val	Gly	Pro	Met
					115			120						125		
20	Leu	Ile	Glu	Phe	Asn	Met	Pro	Val	Asp	Leu	Glu	Leu	Val	Ala	Lys	Gln
		130					135						140			
	Asn	Pro	Asn	Val	Lys	Met	Gly	Gly	Arg	Tyr	Ala	Pro	Arg	Asp	Cys	Val
	145					150					155				160	
25	Ser	Pro	His	Lys	Val	Ala	Ile	Ile	Ile	Pro	Phe	Arg	Asn	Arg	Gln	Glu
					165					170					175	
	His	Leu	Lys	Tyr	Trp	Leu	Tyr	Tyr	Leu	His	Pro	Val	Leu	Gln	Arg	Gln
30					180					185				190		
	Gln	Leu	Asp	Tyr	Gly	Ile	Tyr	Val	Ile	Asn	Gln	Ala	Gly	Asp	Thr	Ile
					195				200					205		

7/8

	Phe Asn Arg Ala Lys Leu Leu Asn Val Gly Phe Gln Glu Ala Leu Lys
	210 215 220
5	Asp Tyr Asp Tyr Thr Cys Phe Val Phe Ser Asp Val Asp Leu Ile Pro
	225 230 235 240
	Met Asn Asp His Asn Ala Tyr Arg Cys Phe Ser Gln Pro Arg His Ile
	245 250 255
10	Ser Val Ala Met Asp Lys Phe Gly Phe Ser Leu Pro Tyr Val Gln Tyr
	260 265 270
	Phe Gly Gly Val Ser Ala Leu Ser Lys Gln Gln Phe Leu Thr Ile Asn
15	275 280 285
	Gly Phe Pro Asn Asn Tyr Trp Gly Trp Gly Gly Glu Asp Asp Ile
	290 295 300
20	Phe Asn Arg Leu Val Phe Arg Gly Met Ser Ile Ser Arg Pro Asn Ala
	305 310 315 320
	Val Val Gly Arg Cys Arg Met Ile Arg His Ser Arg Asp Lys Lys Asn
	325 330 335
25	Glu Pro Asn Pro Gln Arg Phe Asp Arg Ile Ala His Thr Lys Glu Thr
	340 345 350
	Met Leu Ser Asp Gly Leu Asn Ser Leu Thr Tyr Gln Val Leu Asp Val
30	355 360 365
	Gln Arg Tyr Pro Leu Tyr Thr Gln Ile Thr Val Asp Ile Gly Thr Pro
	370 375 380

8/8

Ser

385